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THE GENETICS OF SUSCEPTIBILITY TO LEPROSY

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT

There is strong evidence that genetic factors play a role in susceptibility to leprosy. Although the major histocompatibility complex is known to play a part, it does not account for the whole picture. Recent advances in genetic technology have made it possible to identify loci involved in complex traits. In this thesis I discuss two contrasting approaches: a genome-wide search for the detection of major genes, and candidate gene association studies capable of detecting smaller genetic effects.

242 affected sibling pairs with leprosy were identified in Tamil Nadu in South India. DNA extracted from their blood was used to perform a two-stage genome wide search. Initially 92 families were screened using 293 polymorphic microsatellite markers. A further 96 families were then used to rescreen the markers that showed lod scores suggestive of linkage. Markers on chromosomes 2, 6, 10, 16 and 17 showed increased sharing which could be consistent with the presence of minor genes. These regions include some interesting candidate genes that warrant further investigation.

Association studies were performed on 5 candidate genes using cases and controls from South India, Bengal, and Mali: Interleukins-4 and –10, tumour necrosis factor (TNF), vitamin D receptor, and the natural resistance associated macrophage protein 1 (NRAMP1). An association was found between the NRAMP1 1729+55del4 polymorphism and leprosy type in a population from Mali, mutant homozygotes being susceptible to paucibacillary leprosy whilst heterozygotes were susceptible to multibacillary leprosy (χ^2 =8.88, p=0.00288 OR=5.79{CI 1.46-24.61}). These findings persisted after logistic regression to allow for region and ethnicity. This association was not observed in the South Indian cases and controls. A borderline association was found

between the TNF – 238 polymorphism and susceptibility to leprosy in a South Indian population, cases having an excess of the common allele compared to controls ($2x2\ \chi^2 = 4.12$, p=0.042 OR=1.68{CI 0.98-2.87}).

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ABBREVIATIONS

A	Adenine
ABI	Automated Biosystems Incorporated
APC	Antigen presenting cells
ASP	Affected sib-pair
BCG	Bacille Calmette Guerin
Bcg^r	BCG resistant strain
Bcg^s	BCG susceptible strain
BB	Borderline Borderline leprosy
BL	Borderline Lepromatous leprosy
BT	Borderline Tuberculoid leprosy
C	Cytosine
°C	Degrees centigrade
CAT1	Cationic amino acid transporter 1
CCR5	CC Chemokine receptor 5
CIN1	Cervical intraepithelial neoplasia 1
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'- (5'-chloro) tricyclodecan}-4-yl) phenyl phosphate (a chemiluminescent agent)
CTL	Cytotoxic T lymphocytes
df	Degrees of freedom
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide phosphate
DZ	Dizygotic (twins)

EDTA	Ethylenediamine tetraacetic acid
ENL	Erythema nodosum leprosum
g	Gram
G	Guanine
HLA	Human leukocyte antigen
HIV	Human immunodeficiency virus
HRR	Haplotype relative risk
IBD	Identical by descent
IBS	Identical by state
IDDM	Insulin dependent diabetes mellitus
IFNγ	Interferon – gamma
IL-2	Interleukin2
IL-4	Interleukin-4
IL-10	Interleukin-10
INOS	Inducible nitric oxide synthase
JALMA	Japanese Leprosy Mission in Agra
kD	Kilo Daltons
λ_s	The risk to the sibling of an affected proband of developing the disease compared with the background population risk
L (e.g.53L)	Long PCR programme (defined in Table 2.1)
LAM	Lipoarabinomannam
LL	Lepromatous leprosy
Lod	Natural logarithm of the likelihood ratio ("log of the odds")

MB	Multibacillary
MBP	Mannose binding protein
MDT	Multidrug therapy
MHC	Major histocompatibility complex
µl	microlitre
µM	micromolar
Mins	Minutes
ml	millilitre
mM	millimolar
M. leprae	Mycobacterium leprae
MLS	Maximum Lod score
M. tuberculosis	Mycobacteria tuberculosis
MZ	Monozygotic (twins)
ng	nanogram
NK	Natural killer
NMS	Non medical supervisor
NO	Nitric oxide
Nramp1	Natural resistance-associated macrophage protein 1 (mouse)
NRAMP1	Natural resistance-associated macrophage protein 1 (human)
OR	Odds ratio
PB	Paucibacillary
PIC	Polymorphic information content
PCR	Polymerase chain reaction
PGL-1	Phenolic glycolipid-1

$1,25(OH)_2D_3$	1α,25 dihydroxy vitamin D_3
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
Rpm	Revolutions per minute
s	Seconds
SSC	Sodium chloride/ sodium citrate buffer
SDS	Sodium dodecyl sulphate
SPSS™	Statistical Package for Social Sciences
SSO	Sequence specific oligonucleotide
SSPE	Sodium chloride, sodium hydrogen phosphate and EDTA
T	Thymidine/thymine
TAE	Buffer containing Tris, acetatic acid and EDTA
Taq	Thermostable DNA polymerase (Thermus aquaticus)
TBE	Buffer containing Tris, boric acid and EDTA
TE	Buffer containing Tris and EDTA
Th1	T helper type 1 cells-Subset of CD4+ T
Th2	T helper type 2 cells-Subset of CD4+ T cells
θ	Recombination fraction
T_m	Melting temperature
TMAC	Tetramethylammonium chloride
Tris	Tris hydroxymethyl aminomethane
TNF	Tumour necrosis factor
TT	Tuberculoid leprosy

VDR	Vitamin D receptor
XL(e.g.53XL)	Extra Long PCR programme (defined in Table 2.1)
χ^2	Chi-squared
z_I	The probability of an affected sib-pair sharing i alleles identical by descent

CHAPTER 1

1 INTRODUCTION

1.1 BACKGROUND TO GENETICS AND INFECTIOUS DISEASES

It is likely that infectious diseases have played a major role in the natural selection of humans over the last 5000 years or so (Haldane 1949). With the advent of antibiotics and vaccines it finally seemed possible that we might be able to defeat microbes. However, it was not long before it became apparent that the war was far from over (Ridley 1993). The ever growing problem of multi-resistant bacteria, the resurgence of old infectious diseases such as tuberculosis, and the presence of newly emerging infectious diseases such as AIDS, make it imperative that we use alternative approaches to combat these diseases (Garrett 1995). Even in the presence of rapid advances in molecular biology and vaccine technology, pathogens continue to have the upper hand. At present much hope is pinned on understanding the genetic mechanisms underlying host susceptibility to infection. Once the basic biological processes are known, we may be able to manipulate the host response in such a way that pathology is avoided.

Genetic polymorphisms have been found in various host genes. Both infectious diseases and starvation are likely to have played a major role in determining the frequency of a number of genetic polymorphisms. The differential distribution of polymorphic variations of a gene in a population may give us clues as to the adaptive value of a trait. It has been suggested that in cases where the rarest polymorphism of a gene reaches a frequency of more than 1-2%, this may represent the end result of some

form of selective advantage for the carrier (Motulsky 1960). As well as advantageous effects, polymorphisms may have deleterious effects, which may influence the final frequency of the trait. Both sickle cell disease and cystic fibrosis are examples of such "balanced polymorphisms". Polymorphisms that are beneficial in some diseases may confer susceptibility to other diseases.

There are now numerous pieces of evidence for host factors in susceptibility to infectious disease. There is an extensive list of polymorphisms that control disease severity and outcome for malaria alone. Sickle cell disease and the thalassaemias have reached high frequencies in a number of populations as a result of falciparum malaria. The Duffy blood group, glucose-6-phosphate deficiency, ovalocytosis and the human leucocyte antigen, HLA B53, are just a few other factors found to affect the ability of the malaria parasite to survive in the host (Hill 1996).

In a Brazilian population linkage analysis suggested that 5q31-33 was involved in the intensity of infection with Schistosoma mansonii. A further study on a Senegalese population confirmed this linkage (Marquet et al. 1996) and (Muller Myhsok et al. 1997). This region includes several cytokine genes as well as genes involved in determining T lymphocyte differentiation (Marsh et al. 1994).

Mutations have been identified in the interferon gamma receptor 1 gene that cause susceptibility to atypical mycobacterial infections (Newport et al. 1996) and (Jouanguy et al. 1996). Polymorphisms in the human natural resistance associated macrophage protein, NRAMP1, has been found to influence susceptibility to tuberculosis (TB) (Bellamy et al. 1998) and HLA DR2 plays a role in susceptibility to both TB and leprosy (Rajalingam et

al. 1996) (Todd et al. 1990) (Roy et al. 1997). The –308 polymorphism of the tumour necrosis factor promoter region has been found to influence susceptibility to a number of diseases, including malaria, leprosy and leishmaniasis (McGuire et al. 1994), (Roy et al. 1997) and (Cabrera et al. 1995). Identification of a 32 base pair deletion (Δ32) in the CC-chemokine receptor (CCR5) provides protection from infection with the macrophage trophic form of the HIV virus (Fauci 1996).

It is unlikely that leprosy has played a major role in determining our present day genetic make-up as it is a non-fatal disease (although it could be argued that fertility may have been reduced in the affected population, resulting in an excess of leprosy resistance genes in the population). However, it is possible that leprosy susceptibility genes are shared with a number of other infectious diseases e.g. tuberculosis, another intracellular mycobacterium. The chronic nature of leprosy makes it an ideal infectious disease to study by the affected sibling pair approach (see section 4.3) as both siblings are likely to be alive, as will many of the parents.

Leprosy is an immunological disease, affected individuals falling into different positions of the Th1/Th2 spectrum (see section 1.5.1). Studies of the genetics of leprosy can give answers regarding susceptibility to leprosy type as well as leprosy per se. If we can unravel the genetic mechanisms determining someone's position on the leprosy spectrum, we may get new insights into a large number of other conditions, especially those of an immunological nature. By understanding the basic biological mechanisms of the disease process, avenues towards new therapeutic measures may be opened.

In the remainder of this chapter I will give a brief clinical outline of leprosy,

paying particular attention to the evidence for genetic components to susceptibility, and then discuss the approaches that can be used to study the genetics of complex diseases.

1.2 HISTORY OF LEPROSY

Leprosy has been one of the most feared diseases since Biblical times though many of the diseases referred to as leprosy in the Bible may be the result of an error of translation (McDougall 1996) or misdiagnosis. The first written reference to leprosy was in about 600 B.C in the Sushrata Samhita, an ancient Indian medical treatise. The disease is thought to have spread to China and Japan from other parts of Asia, and was probably introduced into Europe by Greek soldiers returning from Asia in the fourth century B.C. Leprosy was at its most prevalent in Europe between the 10th and 15th centuries A.D., numerous leprosaria being established by wealthy families (McDougall 1996). Sufferers were stigmatised having to warn other people of their approach by ringing a bell or blowing a trumpet. Many people believed that it was a disease cast on people as a result of sin, particularly that of a sexual nature (Brody 1974). The isolation suffered by lepers may have contributed to the gradual decline in cases of disease in Europe, though the Black Death, famines and socioeconomic factors may also have played a role. Despite this speculation, the decline in leprosy remains unexplained

Leprosy was still prevalent in Norway at the end of the last century and it is from this country that much of the early authoritative scientific literature on this disease comes. In 1873 Gerhard Henrik Armauer Hansen confirmed his suspicions that leprosy was a communicable disease when he discovered the leprosy bacillus. His father in law, Daniel Dannielson, was a strong proponent of the hereditary nature of leprosy but

further work by Hansen emphasised the infectious nature of the disease and the genetic theories were largely dismissed and forgotten about (Fine 1981). It has only been over the last three decades or so that strong evidence for genetic factors in susceptibility to this disease have re-emerged (see section 1.7).

1.3 EPIDEMIOLOGY OF LEPROSY

1.3.1 PREVALENCE

From estimates of 10-12 million cases of leprosy worldwide in the 1980s, levels have fallen progressively from 5.5 million in 1991, 3.1 in 1993 and 1.8 in 1995, to 1.15 million in 1997. Over half a million new cases were registered in 1996 of whom 16% were children, and almost 1/3 were multibacillary cases (WHO 1997). 73% of new cases were from India, where the prevalence at the beginning of 1997 was 5.9 per 10,000 and the case detection rate was 44 per 100,000. Much of the change in prevalence is related to an alteration in the definition of a leprosy case that followed the introduction of multidrug therapy (MDT) in the 1980's. Consequently, interpretation of the data is very difficult, but it does appear that incidence rates remain remarkably constant (WHO 1997). In virgin populations prevalence rates as high as 250/1000 have been recorded (Wade and Ledowsky 1952).

1.3.2 GEOGRAPHICAL DISTRIBUTION

Fig. 1.1 shows the geographical distribution of leprosy at the current time. Although leprosy is generally considered to be a tropical disease, it was prevalent in Europe during the Middle Ages, and there were still a large number of sufferers in Norway at the end of

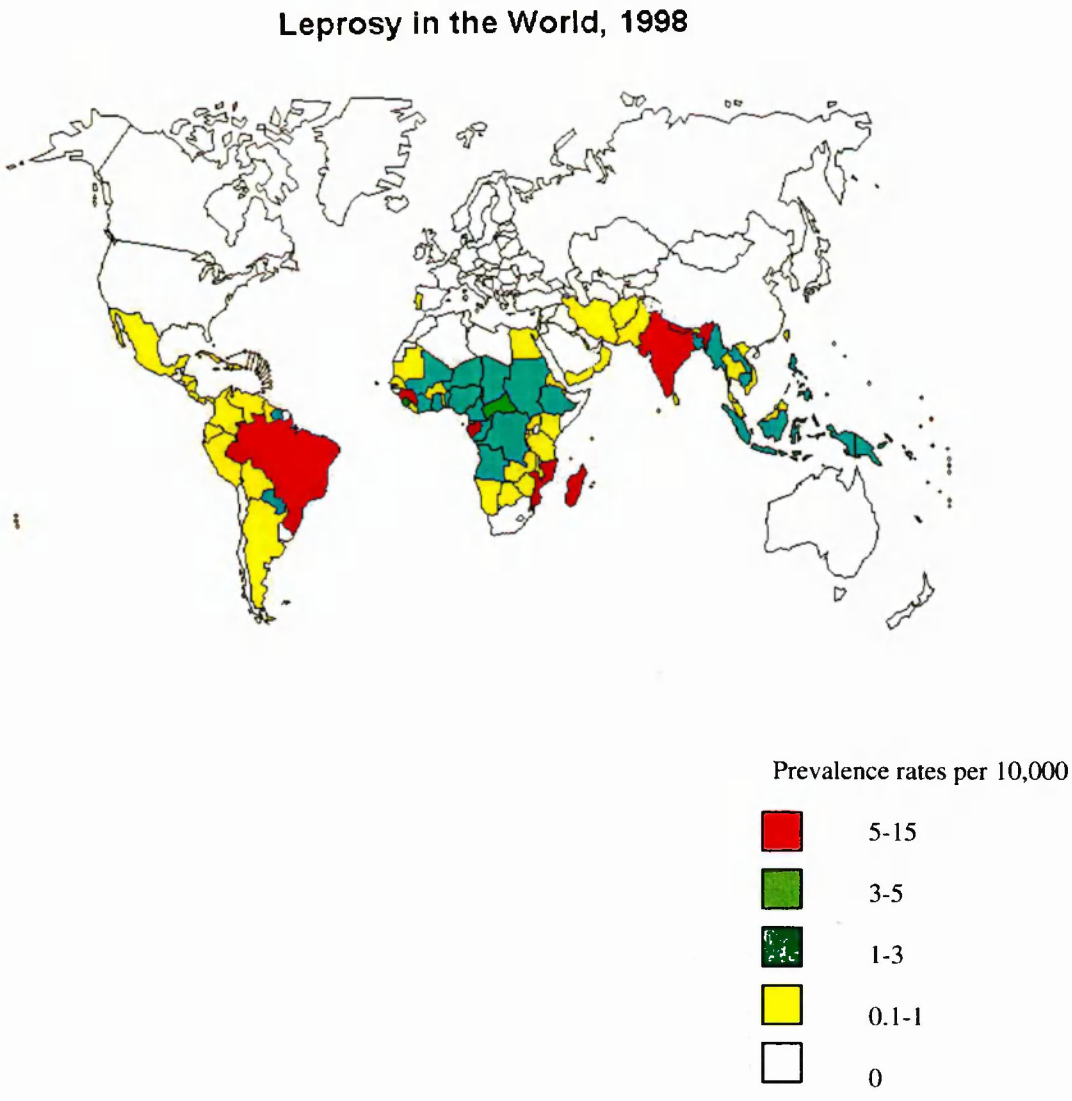

Fig. 1.1 Geographical distribution of Leprosy in 1997 (WHO)

the last century. The proportion of lepromatous to tuberculoid cases is very varied with a tendency for there to be a small percentage of lepromatous cases amongst Africans (4-18%) whereas in Central and South America up to 63 % of cases are lepromatous (Bechelli and Martinez Dominguez 1972). Various complications of leprosy are also frequently regional e.g. leprotic alopecia in Japan and the Lucio phenomenon in South America (Hastings 1994).

1.3.3 AGE DISTRIBUTION

The age distribution of leprosy follows a bimodal pattern with peaks at 10-14 years and a further peak at 35-44, which then plateaus (de Vries and Perry 1985). Leprosy can occur at any age and in virgin populations exposed to leprosy for the first time, leprosy seems to affect all age groups in more or less equal proportions, whereas in populations where leprosy is dying out it tends to affect older adults only (Wade and Ledowsky 1952). Lepromatous leprosy tends to have a later onset than tuberculoid leprosy (Irgens and Skjaerven 1985).

1.3.4 SEX DISTRIBUTION

Males tend to have a higher incidence of leprosy than females once adulthood is reached although there are parts of the world where it is equal or even reversed. This sex difference is more marked in lepromatous leprosy and is also observed in the rate of complications (de Vries and Perry 1985).

1.3.5 OTHER FACTORS INFLUENCING LEPROSY OCCURRENCE

It has frequently been noted that the type and severity of disease differs between two ethnic groups living in the same environment e.g. greater severity of disease in

Europeans noted in both India and South Africa (Spickett 1962). In fact, susceptibility to leprosy appears to be greatest in Caucasians, followed by East Asians, Indians, then Blacks (Bryceson and Platzgraff 1990). Housing, schooling, caste, nutritional status, climate and numerous other factors have been considered to play a role in susceptibility to disease (Fine et al. 1997).

1.4 THE BACTERIOLOGY AND TRANSMISSION OF LEPROSY

1.4.1 BACTERIOLOGY

Leprosy is caused by the acid fast gram positive rod-shaped *Mycobacterium leprae*. It is an obligate intracellular anaerobe with an optimum temperature for *In vivo* growth of 27-30°C (Shepherd 1965). The bacterium remains viable for at least 9 days outside the host (Desikan 1977). *M. leprae* was the first bacterium to be identified, yet it remains elusive in that it cannot be cultured *In vitro*. It has only been in fairly recent years that it could even be cultured experimentally *In vivo*, firstly in the mouse footpad and nude mice, and subsequently in nine-banded armadillos and various primates. Thanks to this we now know that the generation time for *M. leprae* is 11-13 days, the slowest growing bacteria known, and studies on drug sensitivities together with various other characteristics have been possible (Hastings 1994).

Over the last few years the ultrastructure of *M. leprae* has been more clearly defined and the genome sequenced in part (Fsihi and Cole 1995). Because of the difficulties in investigating *M. leprae* we still do not know how it enters the body, what the infecting dose is and whether it is this or host aspects that determine the nature of disease subsequently developing. How many people have a subclinical infection that clears

before it becomes manifest is also unclear. A proportion of people who develop clinically evident disease will self-cure over time. Pathogenicity of *M. leprae* results from its ability to survive intracellularly and to stimulate the host immune system, rather than from the release of toxins.

Unlike other mycobacteria that are found exclusively in macrophages, *M. leprae* is also found in muscle cells, epithelial cells and Schwann cells. The binding protein for entry into Schwann cells is laminin-α2, which is present on the basal lamina (Rambukkana et al. 1997). Fibronectin has also been found to enhance mycobacterial invasion into epithelial and Schwann cells (Schorey et al. 1995). Complement, especially the C2a component, and mannose receptors are involved in entry into macrophages (Schorey et al. 1997).

1.4.2 TRANSMISSION

It seems most likely that bacteria are spread from the respiratory tract of infected individuals. Apart from exceptional circumstances, such as the two cases of leprosy that occurred in tattoos (Porritt and Olsen 1947), it is usually impossible to be certain as to when an infection was acquired. Proposed routes of infection include the respiratory mucosa, the skin (either directly or, more probably, via injuries or bites), transplacental spread and via the gut in the case of breastmilk. Animal models have been used to test hypotheses, but the most important portal of entry in man remains elusive. Since the bacilli survive for at least 9 days in tropical environments (Desikan 1977) it may be that fomites play a role in transmission. Other non-human reservoirs of infection have not been completely ruled out e.g. it has been shown that mosquitoes can act as passive

transmitters of *M. leprae* though their role in the transmission of disease in humans is uncertain (Narayanan et al. 1977).

1.4.3 INCUBATION PERIOD

It has been very difficult to determine the incubation period for leprosy in endemic situations since tests to determine whether infection has occurred are inadequate and the onset of disease is so insidious. The earliest cases recorded were in two children of three weeks of age neither of whose parents had evidence of leprosy though there was a strong family history of the disease in one case (Montestruc and Berdonneau 1954). In this paper they also mention two other cases with extremely short histories of exposure to leprosy and short incubation periods. The longest known incubation period quoted is 40 years (Bryceson and Platzgraff 1990). NCDC data from U.S. war veterans whose exposure to disease was limited to a short period, found incubation periods from 2.9 to 5.3 years for tuberculoid leprosy, and 9.3 to 11.6 years for lepromatous disease (NCDC USA 1970 data quoted in (Hastings 1994)).

Subclinical infection is well recognised. More than 50% of individuals with occupational contact with leprosy for over one year had positive lymphocyte transformation tests in one study (Godal and Negassi 1973)

1.5 THE IMMUNOLOGY OF LEPROSY

The manifestations of leprosy depend upon an interaction between the infecting organisms and the host's immune response to the bacilli. The clinical spectrum of the disease mirrors the immunological response. In lepromatous leprosy, where numerous bacilli are present, there is suppression or absence of a cell-mediated response. The large

antigen load evokes a strong, but often non-specific, antibody response, which is ineffective at clearing the infection. In tuberculoid leprosy there is a very marked T-cell-mediated response which keeps the bacterial concentrations low but can in itself lead to serious tissue damage. The stronger the cell-mediated response in tuberculoid lesions, the more profound and rapid the onset of anaesthesia and autonomic changes, such as hair loss and loss of sweating. Antibody levels are very low or absent at the tuberculoid end of the spectrum. In between these two extremes the borderline forms of leprosy reflect an intermediate level of cell-mediated immunity and antibody response corresponding to their position in the spectrum. This intermediate state is unstable and is liable to up- or down-grading resulting in inflammation that may lead to pathology including sudden neurological deficits (Ridley and Jopling 1966) and (Hastings 1994).

Sieling et al. have suggested that it may be the lipoarabinomannam (LAM), a heteropolysaccharide in the cell wall of *M. leprae* and *M. tuberculosis* that results in the monocyte mediated immunosuppression seen in lepromatous leprosy (Sieling et al. 1993). LAM inhibits antigen processing, macrophage activation and the release of toxic oxygen free radicals. Phenolic glycolipid-1 (PGL-1) may also be a T-cell suppressor (Mehra et al. 1984).

1.5.1 TH1 VERSUS TH2 RESPONSE

T helper cells can be divided into two distinct populations of CD4 cells, Th1 and Th2, according to their function and to the cytokines that they produce. A third category of CD4 cells, Th0 cells, release a mixture of Th1 typical and Th2 typical cytokines. Th1 cells activate cellular immunity and are particularly effective in combating diseases caused

by viruses, intracellular bacteria and parasites. The predominant Th1 cytokines are interferon-γ, interleukin-2, TNF and interleukin-12. Th2 cells enhance the humoral immune response, stimulating B cells to produce antibodies, especially IgE, IgA and IgG1. This type of response is more effective at dealing with free-living bacteria and other extracellular organisms such as helminths. The Th2 cytokines are interleukins 4, 5, 6, 10 and 13. IL-4 and IL-10 have marked T-suppressor activity. IL-4 and 5 regulate B cells, eosinophils and mast cells. Th1 cells downregulate Th2 cells and vice versa, as a result of which there tends to be a polarisation of the final response (Powrie 1993). *M. leprae* reactive T cells from tuberculoid patients are mainly of the Th1 type, whereas Th2 cells predominate in lepromatous lesions. When lepromatous leprosy lesions are injected with Th1 like cytokines there are clear signs of increased cell-mediated immunity and evidence of bacterial degradation (Kaplan 1994).

1.6 CLINICAL ASPECTS OF LEPROSY

1.6.1 THE CLASSIFICATION OF LEPROSY

Leprosy has the most varied clinical manifestations of any infectious disease. Over the years numerous classifications have been established in an attempt to make descriptions easier. These have been based on morphological features, bacteriological, immunological and histopathological findings. The most frequently used classification is the Ridley-Jopling classification which is based on all of the above findings (Ridley and Jopling 1966). This classification divides leprosy into TT (polar tuberculoid), BT (borderline tuberculoid), BB (borderline borderline), BL (borderline lepromatous), and LL (polar lepromatous). Indeterminate leprosy and pure neuritic leprosy are two other

types of leprosy that do not fit neatly into the above spectrum. In the majority of field situations the resources for accurate classification by the Ridley-Jopling method are unavailable. For the purposes of treatment, the WHO have classified leprosy as multibacillary (MB, which encompasses LL, BL and BB) and paucibacillary (PB, which includes TT, BT, Indeterminate and pure neuritic).

1.6.2 CLINICAL SIGNS

The cardinal features of leprosy are anaesthetic skin lesions, thickened nerves and the presence of mycobacteria in slit-skin smears and biopsies. Although there are differential diagnoses for the former two criteria, their presence in a leprosy endemic region is invariably due to leprosy. In the majority of people exposed to *M. leprae* there is either no invasion, or there is sub-clinical infection which resolves spontaneously (Lara and Nolasco 1956). In cases where the disease is not contained, clinical signs and symptoms may not develop for some years and may be akin to post-primary tuberculosis.

The most frequent early finding is of indeterminate leprosy, characterised by poorly defined hypopigmented macules with no diagnostic features. Early tuberculoid lesions appear as hypopigmented or slightly erythematous macules with hypoanaesthesia and typically the involvement of one nearby nerve trunk (see fig. 1.2). Early lepromatous lesions are often numerous but difficult to see until the skin becomes infiltrated when there is a diffuse shininess to the skin, or papules start to appear. Patients may present with anaesthesia or sudden loss of motor function or weakness. More unusual presentations include tenosynovitis, iridocyclitis, nasal stuffiness, oedema, neuritic pain or symptoms of a reaction. The borderline forms of leprosy display characteristics inter-

Fig. 1.2 Photograph of a patient with tuberuloid leprosy.

This shows a depigmented patch of skin just behind the ear, which was anaesthetic on examination. More clearly seen is a grossly thickened greater auricular nerve.

mediate between TT and LL (Ridley and Jopling 1966).

1.6.3 THE COURSE OF THE DISEASE

Early tuberculoid and indeterminate lesions frequently resolve spontaneously. Continuation of the disease may lead to serious nerve damage in tuberculoid leprosy and to chronicity and long-term complications in lepromatous disease. Lepromatous patients are also prone to develop Type 2 reactions (see section 1.6.4). Patients in the borderline regions are unstable and will tend to up- or down-grade with an acute Type 1 reaction and the onset of neurological deficit may be insidious over a number of years.

Lepromatous disease may be virtually imperceptible due to diffuse infiltration, but in advanced cases patients may have leonine facies, destruction of the facial bones, nodules and ulcers, unpleasant smelling nasal discharge, blindness, and deformity from nerve damage and trauma (fig. 1.3). These factors all contribute to stigmatisation. The bacilli can invade internal organs although they do not seem to cause direct damage in these sites. In the long-term amyloidosis and glomerulonephritis may lead to premature death in these patients.

1.6.4 LEPROSY REACTIONS

Reactions constitute some of the major complications of leprosy, particularly now that leprosy is treatable. They can be classified into Type 1 and Type 2 reactions. The former occur in borderline cases of leprosy, the latter in lepromatous and borderline lepromatous cases. Pregnancy, vaccination, acute illness or stress may precipitate both Type 1 and Type 2 reactions.

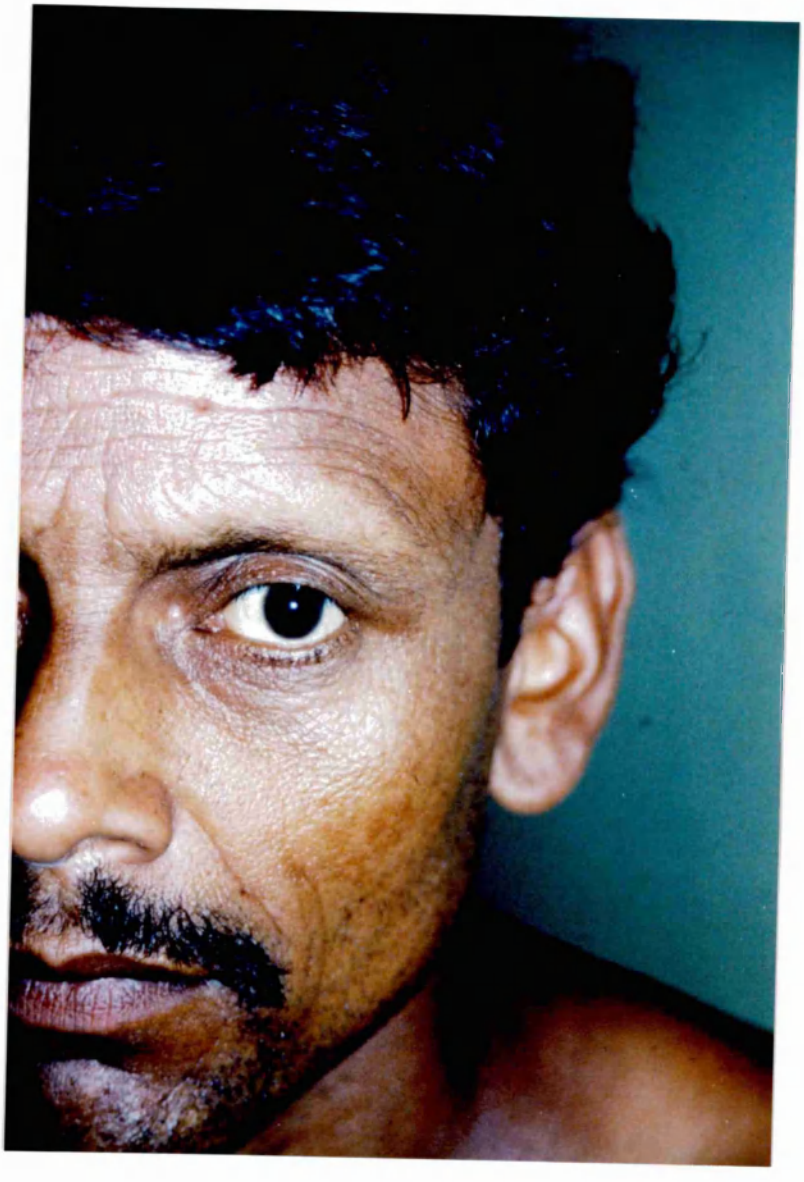

Fig. 1.3 Photograph of a patient with lepromatous leprosy.

There is diffuse waxy infiltration of the skin. Madarosis, or loss of the lateral aspect of the eyebrow, is clearly seen in this patient. He does not, however, have thickening of the earlobes, a common feature of lepromatous leprosy

Type 1 reactions, whether they are due to up- or down- grading, are characterised by increased inflammation in a pre-existing lesion and progression towards one or other end of the leprosy spectrum. Crops of new lesions may appear and nerves become tender and swollen with accompanying neurological signs or symptoms. These reactions can occur in people anywhere along the leprosy spectrum, but most frequently in unstable borderline cases. The underlying basis for the reaction appears to be a sudden increase in cell mediated immunity against *M. leprae* antigens, though what precipitates this remains uncertain. Histologically, there is inflammation with an influx of CD4 cells expressing and producing Th1 type cytokines (Yamamura et al. 1992). It is the production of cytokines that can lead to the rapid onset of nerve damage. As many as 50% of borderline patients will develop a Type 1 reaction during the first 12 months of treatment (Hastings 1994). Roche et al. found that PGL-1 seropositivity and, to a lesser extent, lepromin reactivity were independently associated with increased risk of type 1 reactions ($p<0.0005$ and $p<0.005$ respectively) (Roche et al. 1997).

Type 2 reactions occur in lepromatous and borderline lepromatous cases, the most common clinical manifestation being erythema nodosum leprosum (ENL). This is characterised by multiple erythematous subcutaneous tender nodules, papules and pustules accompanied by fever and constitutional symptoms. These resolve, passing through the discolouration characteristic of bruising, but may leave severe scarring (fig. 1.4). Episodes typically last between a few days and a few weeks but are frequently recurrent and associated with other systemic manifestations, such as fever, malaise, arthr-

Figure 1.4 Photograph of a man who has had Erythema Nodosum Leprosum on numerous occasions.

Note the scarring on his face and arms from recurrent lesions, the moon facies secondary to high dose steroid therapy, and the evidence of neurological damage in both hands with a scar from decompression surgery on the left forearm

algia, iridocyclitis, orchitis, periostitis and tenosynovitis. Treatment with multi-drug therapy is a frequent trigger of type 2 reactions, probably due to the large load of dead bacilli. More than 50% of LL, and about 25% of BL, patients will get a type 2 reaction, although these figures are thought to be declining since the introduction of multi drug therapy (Hastings 1994) and (Becx-Bleumink and Berhe 1992).

The pathogenesis of ENL is thought to be an acute immune complex mediated reactional episode (type III Arthus reaction) due to deposition of antigen-antibody complexes which activate complement resulting in neutrophil chemotaxis (Ridley and Ridley 1983). The toxic products released by the neutrophils result in tissue damage (Naafs 1994). Histologically there is acute inflammation with dilated capillaries, oedema, vasculitis, neutrophil infiltration and fibrinoid necrosis. Histiocytes containing fragmented bacteria are frequently seen (Rao and Rao 1987). Immunoglobulins and complement are found in the lesions on appropriate staining and circulating immune complexes and degradative products of the complement pathway can be detected in the blood.

Traditionally, ENL has been thought of as demonstrating a predominantly Th2 type response (Ottenhoff 1994) and (Yamamura et al. 1992). However, it now appears that the acute episodes may actually represent transient Th1 type responses. Such reactions are accompanied by an increase in cell mediated immunity with an increase in the ratio of T helper to T suppressor cells and a transient increase in responsiveness of peripheral blood lymphocytes to phytohaemagglutinin and mycobacterial antigens (Rao and Rao1987). Once the acute reaction is over the cytokine profile returns to a lepromatous pattern. Filley et al. demonstrated high circulating levels of IL-2 receptors during ENL

and increased levels of agalalactosyl IgG, both of which suggest increased T cell activity. They also demonstrated an effect on B cell regulatory events (Filley et al. 1989). Levels of agalalactosyl IgG are also raised during TB, Crohn's disease and rheumatoid arthritis, but not in the non-reactional state of lepromatous leprosy or sarcoidosis. This suggests that ENL may be triggered by an acute phase response on a background of chronic T cell mediated inflammation (Filley et al. 1989).

1.7 EVIDENCE FOR GENETIC FACTORS IN SUSCEPTIBILITY TO LEPROSY

For centuries people have noted that leprosy tends to run in families. In the latter half of the last century there was much dispute as to whether leprosy was a genetic or environmental disease. When Hansen discovered the leprosy bacillus in 1873, all the previous theories of a hereditary cause for leprosy appeared to be redundant (Fine 1981). It has only been in the last few decades that the possibility of a genetic component in susceptibility to leprosy has resurfaced. The evidence now available shows that both the "germ theorists" and the "hereditarians" were correct. Evidence comes from twin studies, family and community studies, segregation analysis, marker studies and recently candidate gene studies, and anecdotal evidence. Some of these are elaborated below.

1.7.1 ANECDOTAL

There are numerous reports and observations of multiple cases of leprosy within members of the same family (Aredath 1983) and (Anon. B.C.). Racial differences have also been noted, suggesting possible host genetic differences. For example, in Malaya it was noted that one third of leprosy cases in Chinese were tuberculoid whereas three quarters of those in Indians were of this type (Spickett 1962). These racial differences

have since been supported by findings on differential macrophage function *in vitro* between different racial groups (Crowle and Elkins 1990).

1.7.2 TWIN STUDIES

Twin studies are a useful method for dissecting the degree to which environmental and genetic factors play a role. Monozygotic (MZ) twins share the same genes, whereas dizygotic (DZ) twins share only half their genes on average. It is assumed that MZ and DZ share their environments to roughly the same extent if they have been brought up together. If a disease has a strong genetic component, MZ twins would be expected to be more concordant for the disease in question than DZ twins. In a relatively large study of twins with leprosy performed in Bengal, concordance rates for leprosy were 60% in monozygotic (MZ) twins and 20% in dizygotic (DZ) twins. It is of interest that although concordance for leprosy type was similar for both MZ and DZ twins (see Table 1.1), there were 5 out of 37 monozygous sets of twins where the type of leprosy differed. This suggests that host genetic factors are not the only determinants of leprosy type (Chakravartti and Vogel 1973 referred to in Fine 1981). Very similar results were found in a smaller study in South India (Mohammed Ali and Ramanujam 1966). Ascertainment bias is a possibility in both these studies as there were an excess of monozygous over dizygous twins.

1.7.3 SEGREGATION STUDIES

Segregation analysis is a method in which the pattern of inheritance observed in large pedigrees is fitted to a variety of models of inheritance. The model that provides the closest fit is felt to be the most probable explanation for the inheritance pattern. Abel

	62 MZ Cases	Leprosy Type	40 DZ Cases	Leprosy Type
Concordant for disease	37 (60%)		8(20%)	
Concordant for type	32 (86%)	34%LL, 59%TT, 6%Borderline	6 (75%)	33%LL, 67% TT
1 affected	25	24%LL, 72%TT, 4%Borderline	32	25%LL, 63%TT, 13%Borderline

Table 1.1 Results of Chakravartti and Vogel's Twin study in Leprosy

MZ = monozygous twins

DZ = dizygous twins

LL = lepromatous leprosy

TT = tuberculoid leprosy

Borderline = borderline leprosy

and Demenais performed a study on 27 multigenerational pedigrees from Desirade, an island where the incidence of leprosy was particularly high as everyone from the surrounding islands who succumbed to the disease was deported there from 1728 onwards. The results of this study were consistent with an autosomal recessive or codominant major gene controlling susceptibility to leprosy per se and to non-lepromatous leprosy respectively. In this population, assuming a recessive inheritance, the risk of developing leprosy was 62% by the age of 68 in those homozygous for the recessive alleles. They also calculated that the proportion of leprosy cases due to non-genetic factors was 23% in this population (Abel and Demenais 1988). Several other groups have performed segregation analyses and found that susceptibility would be consistent with a recessive model of inheritance e.g. (Haile et al. 1985). It was this evidence of a major gene effect in leprosy that lent support to the performance of a genome screen.

1.7.4 FAMILIAL CLUSTERING

Family clustering has frequently been observed in leprosy, but one of the most difficult aspects in the interpretation of this type of data is differentiating between the shared environmental and the shared genetic factors. Bias can be introduced due to lack of standardisation for family size, different age distributions, household clustering rather than family clustering, and social factors e.g. lepers marry lepers, or stay at home (Fine 1981).

Shields et al. performed a fascinating study on the Karimui of Papua New Guinea, which overcame some of these problems. The social unit of this tribe is the community

rather than the family and polygamy is common. Children spend very little time with their fathers, yet their risk of developing leprosy was increased if they were from a strongly affected kindred, irrespective of whether this was on the mother's or father's side (Shields et al. 1987).

1.7.5 GENETIC MARKERS

The most frequently tested genetic markers are those of the major histocompatibility complex. It is likely that the present diversity of the HLA system has been shaped by selective pressure from infectious diseases as shown by Hill et al's findings of HLA types and malaria (Hill et al. 1991). The cellular immune system is likely to be more important in protecting the host from intracellular infections than the humoral immune system. It is therefore not surprising that numerous studies have been performed on HLA in leprosy. The studies have generated a large number of differing findings (summarised by Todd et al. 1990). Some have shown association between leprosy type and different HLA groups, some with leprosy per se, and others have shown no association whatsoever. The most consistent association has been with HLA DR2 and DQw1 and susceptibility to leprosy per se. Comparisons of these studies are hampered by variations in the classification of leprosy from study to study, frequently small numbers, and poorly matched controls. In the early days typing methods varied enormously.

Todd et al. pooled the data of their own study in Louisiana with other population studies and found that DR2 and DQw1 were significantly associated with leprosy with relative risks of 2.65 and 2.73 respectively, and p values of $1x10^{-8}$ and $3.6x10^{-8}$. When they looked at studies on polar forms of leprosy versus controls they found that both of

the above HLA types were associated with both forms of leprosy. DRw53, DR4 and A24 were also associated in the pooled data even after correction (Todd et al. 1990).

The fact that different associations have been found in different populations would suggest that linkage disequilibrium may be playing a role, and that a gene in close proximity to the HLA region is in fact the leprosy susceptibility locus rather than HLA itself. In favour of a direct role of various HLA types, recent work looking at epitopes from heat shock proteins has shown that although they are recognised by a diverse range of HLA alleles, DR2 is the most efficient restriction element in both tuberculoid and lepromatous disease (Mitra et al. 1997). Risch looked at leprosy data collected by Haile et al. and calculated that the increased risk to siblings over the general population risk for tuberculoid leprosy was 2.38, 1.49 of this being attributable to HLA (Risch 1987) and (Haile et al. 1985). In other words, HLA is insufficient to account for the total genetic component of leprosy susceptibility. This suggests that there are likely to be other genes outside this region that are contributing to the genetic risk.

Numerous genetic markers have been studied for association with leprosy, e.g. ABO blood groups, glucose-6-phosphate dehydrogenase deficiency, dermatoglyphics (Gupta and Tutakne 1986), (Beiguelman 1967) and (Blackwell et al. 1997). Of the other markers studied, results have been varied and inconsistent, reflecting genetic heterogeneity, inappropriate significance levels or poor control groups. Interestingly, colour blindness has been noted to be more prevalent in leprosy patients than in controls in two independent studies (Shwe 1992) and (Ghei et al. personal communication). Shwe examined 697 leprosy patients and 292 healthy controls from Myanmar for colour blindness using Ishihara plates. 7.88% of male tuberculoid leprosy patients and 12.18%

of male lepromatous leprosy patients were red-green or totally colour blind. Only 0.67% of male controls fell into this category and only 1 out of 220 females examined had this problem (an LL case).

1.7.6 GENE IDENTIFICATION

Recently a number of candidate genes have been studied for association with leprosy type and leprosy per se. Roy et al. have looked at the tumour necrosis factor (TNF), vitamin D receptor (VDR), the natural resistance associated macrophage protein (NRAMP1) microsatellite, and mannose binding protein (MBP) in leprosy patients from Calcutta. The –308 TNF polymorphism and the VDR Taq 1 polymorphism genotype are associated with leprosy type in this population. No association was found for either the NRAMP1 microsatellite or mannose binding protein (S. Roy - personal communication and Roy et al. 1997). A transporter associated with antigen-processing (TAP2-B) polymorphism has been found to be associated with tuberculoid leprosy in North India (Rajalingam et al. 1997).

1.7.7 LINKAGE STUDIES

A number of studies have been performed to look for linkage between HLA and leprosy. In family studies the question is whether the affected siblings share their HLA haplotypes more often than would be expected by chance. Non-random segregation of HLA haplotypes was demonstrated amongst siblings with tuberculoid and borderline leprosy from Surinam (de Vries et al. 1976). This segregation was also seen amongst those siblings who both suffered from tuberculoid leprosy, but not in those who had different forms of the disease. Another study by the same group demonstrated

segregation of HLA-DR2 alleles with tuberculoid leprosy (van Eden et al. 1980). Haile et al. demonstrated evidence of linkage of tuberculoid leprosy and HLA in a South Indian population (Haile et al. 1985). Abel et al., on the other hand, failed to demonstrate linkage of leprosy with 5 markers, which included HLA and ABO blood group, in their families from Desirade (Abel et al. 1989). More recently, Blackwell et al. have looked at linkage of a number of markers to leprosy in Brazilian families. They demonstrated that HLA is both linked to, and associated with, leprosy per se (Blackwell et al. 1997).

1.7.8 ANIMAL STUDIES

Animal studies have the advantage of the possibility of specific matings with a rapid turnover, and fewer ethical problems than in humans. Results need to be extrapolated from animals to humans. With the increasing knowledge of genetic sequences, areas of homology between the species can be identified, making some of these assumptions more plausible. The natural resistance associated protein, Nramp1, of mice has been found to affect susceptibility to infections with a number of intracellular organisms including *M. lepraemurium* (see chapter 6).

1.7.9 ADOPTION STUDY

Adoption studies can be particularly useful in assessing the degree to which a disease is influenced by genetic factors. These studies can be combined with twin studies (where numbers allow) to provide particularly interesting results. Their main drawback is that they require large numbers and reliable medical records. There are no studies on leprosy in adoptees, but Sorensen et al. performed a fascinating study on infectious diseases as a whole in Danish adoptees. They found that the risk of a child dying from an infectious

disease was increased more than 5 fold if one of their biological parents had died of an infectious disease before the age of 50, whereas the risk was close to unity if the adoptive parents did so. The reverse was true for cancer. This suggests that susceptibility to infectious diseases has a major genetic component (Sorensen et al. 1988).

1.7.10 SEX RATIO

The excess of males affected by leprosy in most populations may be due to ascertainment bias. However, it is interesting to note that similar findings have been found in tuberculosis (MRC 1992) and anecdotally in other infectious diseases. Furthermore, Bellamy et al. have recently found a possible linkage to the X chromosome in a genome wide search for tuberculosis susceptibility genes in the Gambia and South Africa (R. Bellamy - personal communication).

1.7.11 OTHER FACTORS DETERMINING CLINICAL EXPRESSION OF DISEASE

It is likely that the majority of people exposed to *M. leprae* mount an adequate immune response so that the disease is never manifest (Godal and Negassi 1973). However some people will get infected and the degree to which their immune system controls the infection will determine the manifestations of the disease. The bacillus is not very toxic and large numbers of bacteria can be tolerated with very few sequelae, most of the pathology being due to the host's own immunological response. A large number of factors have been implicated in determining the host response to infection.

Various host factors play a role in determining the response to infection. Nutritional status at the time of infection may be important. Zinc deficiency results in a switch from a Th1 to a Th2 type response (Sprietsma 1997) and a number of vitamin deficiencies

have been associated with leprosy (Foster et al. 1988). Mitra et al. have evidence that suggests that the MHC phenotype can modulate the Th1 vs. Th2 profile that the patient develops against leprosy (Mitra et al. 1997). Interactions with genes other than the MHC may play a role. Specific epitopes may push the response to one end of the spectrum or the other e.g. Liew et al found that a specific 10-mer epitope resulted in a Th2 response to Leishmania major in mice (Liew et al. 1990).

The hormonal environment at the time of infection may be important in determining the clinical picture. Certainly, there is an increased rate of complications and relapses during pregnancy, and there are changes in the epidemiological pattern seen at puberty as compared to childhood (de Vries and Perry 1985). Both age at exposure to the bacillus and birth order have been implicated in determining the form of disease that develops. It has been suggested that earlier infection may predispose to the tuberculoid form of disease (Koumantaki et al. 1987). Although the evidence is weak, this theory is supported by the higher incidence of tuberculoid disease in highly endemic areas than in non-endemic areas (Fine 1982).

Parasite factors including infecting dose and antigenic load, may determine the host response (Howe et al. 1995). Bancroft et al. found that Balb/K mice, that are usually resistant to nematode infections, become infected if the initial infecting dose was less than 40 eggs (Bancroft et al. 1994). This may be because a Th1 response is induced by a low antigenic load (Hosken et al. 1995). Lipoarabinomannam has been proposed as a virulence factor in mycobacterial disease (Chan et al. 1991). It appears to downregulate macrophage effector function and inhibits the release of toxic oxygen free radicals (Kaplan et al. 1987). PGL-1 of *M. leprae* may also trigger T cell suppressor cells (Mehra et

al. 1984). Nitric oxide (NO) is a potent effector of macrophage stasis/cidal activity. Prostaglandins and transforming growth factor β inhibit NO production. Both interferon gamma and tumour necrosis factor enhance macrophage phagocytic capacity and possibly mycobacterial killing *in vivo* (Bermudez 1988). There may be other mechanisms that influence the production and effectiveness of reactive intermediates.

Recent, co-existing or superimposed infections may alter the host immune response. Parronchi found that the presence of concurrent atopic allergy affected cytokine production by *M. tuberculosis* infected specific clones of macrophages (Parronchi et al. 1991). Previous exposure to other mycobacteria and to BCG may affect the host response to infection (Rook et al. 1981). Antigen-antibody complexes promote a Th2 type response, though they will enhance a Th1 response as long as they lack the ability to crosslink Fcγ receptors on the cell surface (Berger et al. 1996).

The route of infection and the infection type may determine whether there is predominantly a Th1 or a Th2 response. For example, the intradermal route of infection in the mouse footpad sensitises the mouse to *M. lepraemurium*, whereas the intravenous route tolerises the mouse (Shepherd et al. 1982).

It has been found that there is diurnal variation in the interferon- gamma (IFN-γ): IL-10 ratio with a high ratio in the morning corresponding to the cortisol dip and the peak in plasma melatonin levels. Administration of 25mg of cortisone produced > 70% decrease in the ratio. Because of the opposing effects of IFN-γ and IL-10 on the immune system the timing of infection may determine the subsequent immune response that

develops (Petrovsky and Harrison 1997).

Host genetic factors may affect infection in various ways. Differences in expression of cell receptors to which bacteria or viruses may attach may affect host susceptibility e.g. *M. tuberculosis* gains access to macrophages by binding to C3 and Fc receptors on the cell surface prior to phagocytosis (Walker and Lowrie 1981). *M. leprae* can evade various host defence mechanisms e.g. the production of NH_4 increases resistance to phagosome acidification and there are mechanisms by which phagosome maturation and the fusion of phagosomes with lysosomes are prevented (Schlesinger and Horwitz 1990), (Clemens and Horwitz 1995) and (D'Arcy Hart and Young 1991).

1.7.12 HOST SUSCEPTIBILITY TO LEPROSY

When considering susceptibility to leprosy, there are a number of stages to the infection that need to be considered and may provide clues as to the genetic mechanisms involved. These are outlined below. Assuming exposure, the initial acquisition of infection may involve cell receptors as with CCR5 in HIV infections. In leprosy C3 and Fc receptors on macrophages are used for attachment prior to phagocytosis. Genetic polymorphisms in these could well affect susceptibility to infection.

The next hurdle for the organism is establishing infection. Primed macrophages may rapidly destroy bacteria that enter the cell e.g. Nramp1 in bcg^r mice seems to be active in rapid priming of the macrophages, such that bacteria entering the cells are rapidly destroyed. Nitric oxide synthase is important in controlling the intracellular growth of *M. leprae* (Adams et al. 1991). There are known polymorphisms in iNOS that would be worthwhile considering as candidate alleles for leprosy susceptibility (D.Burgner –

personal communication). Both interferon gamma and tumour necrosis factor enhance macrophage phagocytic capacity and possibly mycobacterial killing *in vivo* (Bermudez 1988).

In leprosy, disease is more closely related to host response than to mycobacterial virulence. Thus it is the bacteria's ability to survive in host cells and to activate the immune system rather than direct cellular damage that contributes to pathology. Infection may be progressive or quiescent. The cytokine response is probably particularly important at this stage, Th1 responses favouring clearance of the organism, Th2 responses allowing excessive growth of bacteria (see section 1.5.1).

Various host factors may keep the disease quiescent as occurs in tuberculosis infections. When conditions alter e.g. the host becomes immunosuppressed, the disease may become active again. Resistance to reinfection may depend on the initial immunological response mounted by the individual and the rapidity with which memory cells mount a response. Previous infection with similar organisms may mean that such a response can be mounted on first exposure to the organism, which may be sufficient to prevent infection. Various environmental mycobacteria and vaccines such as BCG may share sufficient epitopes that the host can keep an infection with leprosy at bay if they have had previous exposure to these factors.

1.8 STRATEGIES FOR MAPPING INFECTIOUS DISEASE SUSCEPTIBILITY GENES

In this study I used a two-pronged approach to detect leprosy susceptibility genes: a genome wide search to look for major susceptibility loci, and some candidate gene

association studies in the hope of detecting some smaller effect genes. Below I give a brief outline of these two methods along with 2 other approaches that have not been applied in the current work.

1.8.1 ASSOCIATION STUDIES

Association, or case control, studies can be used to compare the frequencies of polymorphic alleles between unrelated affected and unaffected individuals from a population. Candidate genes are genes that are suspected to play a role in susceptibility to a given disease because of an *a priori* knowledge of their function. An allele A of a candidate gene is said to be associated with the trait if it occurs at a significantly higher frequency in the affected individuals than in the controls. Analysis of alleles is performed using a 2 x 2 χ^2 test, and analysis of genotypes using a 3 x 2 χ^2 test. The most important aspect of this type of study is to have a well-matched control population. It is a powerful method provided the marker is close to the disease allele and a large enough population is examined. A major limitation is that studies can only be performed for genes that have already been identified and in which polymorphisms are known. Candidate genes are chosen on a best-guess basis, but there are frequently a large number of potential candidate genes for any given trait, of which very few may prove to be associated.

An association may be true and the allele may be the actual cause for the disease or may be in linkage disequilibrium with the disease causing allele. Linkage disequilibrium is the term applied when haplotypic combinations of alleles at different loci occur more frequently than would be expected by random association. This will occur in situations where most people who possess the trait share a common ancestor from whom they

have inherited the mutation alongside the susceptibility gene. This could be due to a population bottleneck, population admixture where allele frequencies in the populations were different, genetic drift, a new mutation, or selective advantage of one of the alleles (Weeks and Lathrop 1995). If the marker is tightly linked to the disease causing allele, the association will be strong and will persist over many generations. Because the marker allele is close enough to the true disease allele, there has been insufficient time for recombination events to erode the association, so the two are inherited together in the majority of cases. For two loci with recombination fraction θ, the initial linkage disequilibrium is reduced by $(1- \theta)^n$ after n generations. It has been estimated that carriers of an allele that coalesced 60 generations ago share 5cM of DNA. This explains the common occurrence of shared genomic areas around a rare mutated allele (van der Meulen and te - Meerman 1997). The particular allele in linkage disequilibrium may vary from one population to another. A third reason for finding an association between an allele and a disease is population artefact due to non-homogeneity of the population (Lander and Shork 1994) e.g. Pima Indians and the Gm locus (Knowler et al. 1988).

The power of association methods depends upon a number of factors. These include the degree of linkage disequilibrium between the marker and the disease locus, and the recombination fraction between the two. Other factors include the frequency of the mutation, the increase in risk due to the allele, and the penetrance of the different genotypes (Weeks and Lathrop 1995). An advantage of case control studies over genome-wide screens is the speed with which they can be performed and analysed, and the relatively low cost. They can detect disease loci that would be missed by linkage analysis (Greenberg 1993).

There are two approaches that can overcome the problems of population stratification that occur in case control studies:

i) Haplotype relative Risk Method (HRR)

The problems associated with having to find a well matched control that are encountered in association studies, are overcome in the HRR method by using an internal control. Nuclear families are used and the "control" is derived from the non-inherited parental alleles (fig. 1.5). This is a non-parametric method. A 3 x 2 table can be constructed of the presence or absence of the allele in question in cases versus "controls" and a χ^2 test performed. Various statistical tests are applicable (Schaid and Sommer 1994). The ratio of discordant pairs provides an estimate of the relative risk (Khoury 1994). It may be obvious looking at the results that a disease is dominant or recessive, and it may even be possible to see the effects of imprinting by examining whether the alleles originate from the mother or father (Schaid and Sommer 1994).

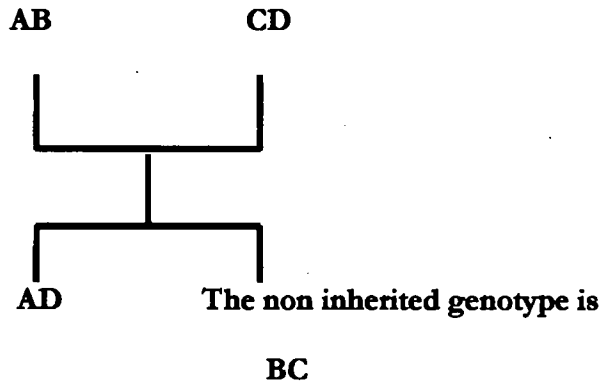

Fig. 1.5 Demonstration of "virtual control" used in the Haplotype Relative Risk Method

There are two main disadvantages to this method. Firstly, because two parents need typing there is 50% more work involved than in a traditional case control study. Secondly, in any disease in which the onset is late there may be problems recruiting parents. The HRR method has been used successfully in establishing an association between attention-deficit hyperactivity disorder and the dopamine transporter locus (Gill et al. 1997).

ii) Transmission Disequilibrium Test

This is a powerful test that has been successfully applied, for example, in the study of diabetes susceptibility loci (Copeman et al. 1995). Parents heterozygous for the allele of interest are considered and the frequency of transmission of the two alleles to their affected offspring is examined. If there is no association, the transmission of each allele would be expected to be 50%. If there is a significant deviation from this, there is evidence of association. It is a useful test to apply in cases where linkage has not been detected, but an association has been found as it directly tests for linkage and is unaffected by population stratification (Spielman et al. 1993). The advantage of this method is that it is unaffected by population stratification, yet it is simpler to perform than linkage studies as multiple affected family members are not required. The disadvantage is that linkage can only be detected if there is association due to linkage disequilibrium between the marker and the locus. Significant results may be artefactual due to meiotic segregation distortion, which would occur in both affected and unaffected children. Provided unaffected children are available this can be excluded.

1.8.2 GENOME-WIDE SEARCHES

It is likely that susceptibility to leprosy is a complex trait with several susceptibility loci that interact with one another and with the environment to determine disease outcome. The evidence from segregation analyses lends support to a major gene effect. It has only recently become feasible to attempt to look at the genetics of this type of disease in a systematic way.

In this approach the entire genome is systematically screened for linkage of major susceptibility loci to regularly spaced markers. It is an expensive and labour intensive method, but, provided the markers are suitably spaced (up to 20cM), it will identify major genes (Terwilliger 1995). It has less power than the candidate gene approach, but is more systematic and has been used effectively in identifying type 1 insulin dependent diabetes loci (Davies et al. 1994). Traditionally, and in our study, people have genotyped both parents and at least two affected siblings. Various approaches have been used in order to try to increase efficiency. Hauser suggested initially screening the affected siblings alone, halving the initial number of genotypes that need to be performed. This is followed by typing additional family members for the markers that produce lod scores of more than 1, together with nearby surrounding markers (Hauser et al. 1996). A simulation study was performed for a disease where the λs of the locus was 2.5. This found that the power of a genome screen to detect a lod score equal to or greater than 2.3, was 99.9% if 96 families were examined for 273 markers at an average spacing of 20cM (Davies et al. 1994).

1.8.3 ANIMAL MODEL HOMOLOGUES

Animals can be bred much more easily than humans and backcross experiments performed so that known genotypes can easily be produced and studied. The animals can

be kept in conditions that maximise the importance of the underlying genetic factors. Because genetic heterogeneity can be eliminated, far more complex traits can be studied in animals than humans. This may help in establishing the physiological and biochemical basis for various mammalian diseases. Dense maps are now available for mice and rats. Numerous diseases have being investigated using quantitative trait locus mapping, some of which have direct human parallels. For example, the finding of linkage of hypertension in rats to the angiotensin converting enzyme led to the establishment of the role of angiotensinogen in human disease (Crabbe et al. 1994) (Tanksley 1993). Using knockout mice, it should be possible to gradually dissect out polygenic causes of different traits.

Unfortunately it is not always possible to extrapolate findings in one species to another. Another problem is that defects are often noted in inbred strains of mice that are in sterile environments. It is possible that if they were in the wild, these strains would never have survived, so the likelihood of a human homologue is very small. Examples of human homologues of mice genes are the IDDM1 and IDDM7 genes causing susceptibility to insulin dependent diabetes (Copeman et al. 1995) and (Todd et al. 1987). After many years of speculation, the human homologue of the mouse natural resistance macrophage associated protein, NRAMP1, is now coming up trumps in a number of diseases (see chapter 6).

1.8.4 POPULATION AND FAMILY STUDIES

Population studies and family studies can be used to investigate the effects of genetic polymorphisms on susceptibility to various diseases. Family studies have the advantage

of eliminating artefacts of population heterogeneity and linkage disequilibrium. They also give extra information about the inheritance, penetrance and frequency of disease susceptibility genes. The disadvantage of selected multicase families is that they may be non-representative of the patient population.

1.9 OUTLINE OF WORK PERFORMED IN THIS THESIS

In view of the strong evidence for genetic factors in leprosy, in particular that from segregation studies pointing towards a major gene effect, we decided to perform a genome-wide search in leprosy-affected sibling pairs. We also performed a few association studies on candidate genes in order to see if they had any role in the disease. The collection of samples, techniques employed, rationale for testing the candidate genes, results and conclusions are discussed in the following chapters.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 OVERVIEW OF METHODS USED

Two methods were used in the work for this thesis. Firstly a two-staged genome-wide search for major leprosy susceptibility loci was performed. Families were identified in which there were at least 2 siblings with a history of proven leprosy. Blood was collected from the siblings and their parents and DNA extracted. In the first instance 92 families were screened using 293 fluorescently labelled microsatellite markers. The microsatellites were amplified by the polymerase chain reaction (PCR), pooled into sets of non-overlapping size ranges, and run with a size marker through polyacrylamide gels on 373 ABI sequencers. The products were sized to within 1 base pair using the programmes Genescan Analysis™ and Genotyper™. Final analysis of the families was performed using GAS™ and Sib-Pair™, computer programmes that perform χ^2-tests and calculate lod scores based upon the observed and expected allele sharing patterns. Multi-point analysis was also performed using Mapmaker Sib™. Markers with lod scores or p-values suggestive of possible linkage were screened in the second stage of the study using a further 96 families.

Secondly, case-control studies of candidate gene polymorphisms were undertaken. The region of interest was amplified by PCR, the product was blotted onto nylon filters, and the filter probed for the different alleles with sequence-specific oligonucleotides (SSO). The SSO's were pre-labelled with digoxigenin (DIG) (produced

by Boehringer Mannheim). Following treatment with an anti-DIG antibody attached to a phosphatase enzyme (anti-DIG-AP), CSPD was added to the filters. CSPD is a chemiluminescent agent that is a substrate for the phosphatase. If the DIG-labelled probe had attached to the filter, chemiluminescence was produced following incubation with CSPD. This was easily detected by exposing the filters to X-ray film, after which scoring of the samples could be performed.

2.2 GENERAL METHODS

(see section2.4 for details of solutions used)

2.2.1 DNA EXTRACTION FROM WHOLE BLOOD

5-9 ml of blood was collected in EDTA tubes and DNA was extracted on the same day using Nucleon II ™ kits (Scotlab). Red cell lysis was performed by adding Nucleon Reagent A (at a volume 4 times that of the blood) to the blood and mixing the solution for 4 minutes at room temperature. The solution was then centrifuged for 4 minutes at 1300g and the supernatant discarded into Virkon. 2ml of Nucleon Reagent B was added to the white cell pellet, admixture and lysis being encouraged by briefly vortexing the tube. The solution was transferred to a 15ml tube and deproteinisation performed by adding 500µl of 5M sodium perchlorate solution and mixing the tube for 15 minutes at room temperature. The tube was then placed in a water bath at 65°C and shaken for 25 minutes. DNA was extracted by adding 2ml of chloroform that had been stored at minus 20°C, and mixing the tube for 10 minutes. 300 µl of Nucleon silica suspension (provided in the kit) was added and the solution centrifuged at 1300g for 3 minutes. This produced a biphasic solution separated by a thin layer of silica solution,

with a lower organic phase, and an upper DNA containing phase. The upper phase was carefully transferred to another tube. If some of the silica solution had also been transferred, it was re-centrifuged and the supernatant transferred to a new tube. The DNA was then precipitated by adding it to approximately twice the volume (usually 7ml) of 100% ethanol at 4°C. The DNA was transferred to an eppendorf tube, allowed to air dry, and then dissolved in 10^{-4} x TE buffer. Following extraction, samples were stored at 4°C or -20°C depending on the availability of facilities, and transportation back to the UK was performed on ice. Samples to be used in the genome screen were diluted further in 10^{-4} x TE buffer to reach a roughly uniform concentration of 170-230ng/µl. Some of this was further diluted with distilled water to reach a concentration of approximately 10ng/µl and stored in 96 well deep – well plates at 4°C in a set format for convenience of use. The other samples were stored at -20°C once back in the UK at concentrations ranging between 200 – 1000ng/µl.

During the second visit to India, the prolonged mixing steps at the sodium perchlorate and chloroform stages were replaced by fully inverting the tubes a minimum of 7 times, and the water-bath incubation step was omitted.

2.2.2 MEASUREMENT OF DNA CONCENTRATIONS

DNA concentrations were measured using a Hoefer fluorimeter. The method is based on the binding of a fluorescent dye (Hoescht 33258) to DNA. Calf thymus DNA at a concentration of 1000ng/ml was used to calibrate the fluorimeter. This allowed accurate measurement of DNA concentrations between 100-2000ng/ml. Each sample was then checked in turn. If the concentration was greater than 2000ng/ ml, it was

diluted until it was within the range.

2.2.3 POLYMERASE CHAIN REACTION FOR AMPLIFICATION OF DNA

The Polymerase Chain Reaction (PCR) method was used to amplify the deoxyribonucleic acid (DNA). Regions lying between two primers were amplified in an exponential way, by repeated cycles of temperature changes that allowed denaturation, extension and annealing of the DNA strands in the presence of appropriate reagents. The primers were chosen according to the area of interest, their estimated melting temperatures, T_m, their length, a lack of secondary structure and a lack of complementarity of the 3' ends either between or within individual primers. Computer programs are now widely available to aid in the selection of specific primers, although in most cases other groups had already identified suitable primers.

Initially Hybaid PCR machines were used for thermal cycling in this project. MJ machines gradually became more readily available during the course of the project, which speeded up the process. The annealing temperature varied with the reaction (generally between 49-61°C), but denaturation temperatures of 94 – 95°C, and extension temperatures of 72°C were used.

A thermostable DNA polymerase Taq (Thermus aquaticus) was used to drive the reaction. In general a standard buffer solution was used with a standard concentration of dNTPs (deoxynucleotide phosphate) and primers. The magnesium concentration was varied between 0.5 – 5 mM in order to optimise the PCR product. The annealing temperature chosen was usually a few degrees below the calculated T_m of the primers, after which optimisations were performed at a variety of temperatures and magnesium concentrations. If the PCR did not work with the standard buffer, further experiments

were performed using the Stratagene™ set of buffers, in order to find the most suitable conditions. Occasionally glycerol or DMSO was added to improve the specificity of the product. 25µl reactions were used for the case-control studies involving sequence-specific oligonucleotides, and 10µl reactions for microsatellite studies. 96 well polycarbonate plates were used and the reaction solutions covered with 1 drop of mineral oil.

The success of reactions was assessed on 1-1.5% agarose gels. Once a PCR had been successfully optimised using a small number of samples, large-scale amplification was performed. Reaction conditions for the candidate genes studied are listed in tables 2.1 to 2.5.

2.2.4 AGAROSE GEL ELECTROPHORESIS

1-1.5% agarose gels were used to check the successful amplification of DNA by PCR. Small gels (60 sample capacity including marker dye) were made from 100ml of TBE (TAE used from mid 1997) with 1 – 1.5g of agarose. This was heated in a microwave oven for 3 minutes until the solution was clear. It was cooled to about 60°C under running tap water after which 5 µl of ethidium bromide was added. This was mixed in thoroughly by swirling the bottle and the solution was then poured into a pre-prepared gel-tray and allowed to set at room temperature for about 20 minutes before use. Larger gels with capacity for 3 x 96 well plates plus markers were made using four times the above amounts.

3 µl of loading dye (0.25% bromphenol blue) was added to 5 µl of DNA product and loaded onto the gel. 3 µl of a ΦX174 molecular weight marker (Pharmacia) with

Gene	Primers	PCR Conditions	Probes (stringency temp)
IL-4	5'-ACT AGG CCT CAC CTG ATA CG-3' 5'GTT GTA ATG CAG TCC TCC TG-3'	25µl reaction: 1 x Ammonium Buffer, 4.5mM $MgCl_2$, 0.2mM dNTPs, 1µM primers, 0.5mM spermidine, 3%DMSO, 1 unit AmpliTaq Hybaid cycler : Hot start: 95°Cx300s, 38x{94°C x60s,57°C x45s,72°C x600s}	5'-GAA CAT TGT CCC CCA GTG-3' (52°C) 5'- GAA CAT TGT TCC CCA GTG-3' (51°C)
IL-10 – 592	5' CTG GCT CCC CTT ACC TTC TAC ACA 3' 5'TGG GCT AAA TAT CCT CAA AGT TCC 3'	25µl reaction: 1 x KCl Buffer, 2mM $MgCl_2$, 0.2 mM dNTPs, 2 µM primers, 1 unit of Taq gold MJ cycler: 94°Cx 14 mins. 35x{94°C x 10s, 58°C x 20s, 72°C x 30s} then 72°C x 2 mins.	5'-CGC CTG TCC TGT AGG-3' (43°C) 5'-CGC CTG TAC TGT AGG-3' (57°C)

Table 2. 1 PCR conditions used in case-control studies

Gene	Primers	PCR Conditions	Probes (stringency temp)
IL-10 – 1082	As for IL-10-592	As for IL-10-592	5'-TTT GGG AGG GGG AAG-3' (48°C) 5'-TTT GGG AAG GGG AAG-3' (42°C)
IL-10 microsat-ellite	5'-GTC CTT CCC CAG GTA GAG CAA CAC TCC -3' FAM 5'-CTC CCA AAG AAG CCT TAG TAG TGT T-3'	15µl reaction: 1x KCl Buffer, 1mM $MgCl_2$, 0.2 mM dNTPs, 6µM primers, 0.5 units of Taq gold MJ cycler: 94°C x 15 mins. 38x{95°C x 1s, 53°C x 20s, 72°C x 5s} then 72°C x 2 mins.	N/A Diluted 1in 40 before loading onto ABI gel
NRAMP Intron4	5'-TCT CTG GCT GAA GGC TCT CC-3' 5'-TGT GCT ATC AGT TGA GCC TC-3'	25µl reaction: 1x KCl Buffer, 1mM $MgCl_2$, 0.2 mM dNTPs, 2 µM primers, 1 unit of Taq gold MJ cycler: 94°C x 15mins, 38x{94°C x 15s, 58°C x 30s, 72°C x 45s}, then 72°C for 2 mins.	3'-TTG GGG GGC CTG GAC-5' (53°C, SSC) 3'-TTG GGG GCC CTG GAC-5' (48°C, SSC)

Table 2.2 PCR conditions used in case-control studies (continued)

Gene	Primers	PCR Conditions	Probes (stringency temp)
NRAMP TGTG	5'-GCA TCT CCC CAA TTC ATG GT-3' 5'-AAC TGT CCC ACT CTA TCC TG-3'	25µl reaction: 1x KCl Buffer, 1mM $MgCl_2$, 0.2 mM dNTPs, 2 µM primers, 10% glycerol, 1 unit of Taq gold MJ cycler: 94°C x 14mins, 5x{94°C x 15s, 58°C x 30s, 72°C x 30s} then 32 x {94°C x 15s, 53°C x 30s, 72°C x 30s), then 72°C x 2mins	5'-CTG GAT GTG GAG GGG-3' (51°C) 5'-TGC TGG AGA GGG GGC-3' (48°C)
NRAMP microsat-ellite	5'-TTC CGC ATT AGG GCA ACG AG-3' 5'-TTC TGT GCC TCC CAA GTT AGC-3'-TET	15µl reaction: 1x KCl Buffer, 2mM $MgCl_2$, 0.2 mM dNTPs, 6µM primers, 0.5 units of Taq gold MJ cycler: 94°C x 15 mins. 38x{95°C x 15s, 55°C x 20s, 72°C x 5s}, then 72°C x 2 mins.	N/A Diluted 1in 40 before loading onto ABI gel

Table 2.3 PCR conditions used in case-control studies (continued)

Gene	Primers	PCR Conditions	Probes (stringency temp)
TNF –238	5'-CAA ACA CAG GCC TCA GGA CTC- 3' 5'-AGG GAG CGT CTG CTG GCT G-3'	25µl reaction: 1 x KCl Buffer, 2.5mM $MgCl_2$, 0.32 mM dNTPs, 1.6 µM primers, 1 unit of Taq gold MJ cycler: 94°Cx 14 mins. 35x{95°C x 10s, 60°C x 20s, 72°C x 30s} then 72°C x 2 mins.	5'- CTG CTC CGA TTC CGA G-3'(56°C) 5'-CCT CGG AAT CAG AGC AGG G-3' (58°C)
TNF – 308	As for TNF – 238	As for TNF –238	5'-AGG GGC ATG GGG ACG GG-3' (61.5°C) 5'-AGG GGC ATG AGG ACG GG-3' (61.5°C)

Table 2.4 PCR conditions used in case-control studies (continued)

Gene	Primers	PCR Conditions	Probes (stringency temp)
VDR	5'-CAG AGC ATG GAC AGG GAG CAA G-3' 5'-GGT GGC GGC AGC GGA TGT A-3'	1x Ammonium Buffer, 2mM $MgCl_2$, 0.2 mM dNTPs, 1 μM primers, 10% glycerol, 1unit of Taq gold MJ cycler: 94°C x 14 mins. 35x{94°C x 30s, 49°C x 30s, 72°C x45s}	5'-GCG CTG ATT GAG GCC ATC-3' (65.5°C) 5'-GCG CTG ATC GAG GCC ATC-3' (63.5°C)

Table 2.5 PCR conditions used in case-control studies (continued)

fragment sizes of 72, 118, 194, 234, 271, 310, 603, 872, 1078 and 1353 base pairs was always run in at least one well per row of products. Electrophoresis of the gel took place in a tank filled with TBE solution (TAE from mid 1997) with a current of 100 volts for approximately 20 minutes. The presence of a single product band of the correct size could then be confirmed by examining the gel under ultraviolet light (Eagle Eye, Stratagene).

2.3 METHODS SPECIFIC TO SEQUENCE SPECIFIC OLIGONUCLEOTIDE PROBING

2.3.1 TRANSFER OF AMPLIFIED DNA TO NYLON FILTER USING DOT BLOTTING

Once amplification of the samples for candidate gene studies (except those of microsatellites) had been successfully performed, the product was transferred to a nylon membrane for probing. Initially 10 µl of the product was rendered single stranded by adding it to 100 µl of a denaturing solution incubated on ice. The denaturing solution was made of 86 µl of TE, 6 µl of 0.5mM EDTA at pH 8.0, and 8 µl of freshly made 6M NaOH. After 10 minutes incubation, the reaction was neutralised with 100 µl of 2M ammonium acetate. A positively charged nylon membrane was placed in a Hybridot manifold on top of 2 moistened pieces of Whatmann paper, and moistened with distilled water. The lid of the manifold was screwed finger-tight, the suction turned on, and 100 µl of 1M ammonium acetate solution was put into each well. Once this had been completely sucked through the filter, the denatured PCR products were transferred to the filter, followed by 200 µl of 1M ammonium acetate. The product was fixed to the filter by UV crosslinking followed by baking at 80°C for 2 hours in an oven.

2.3.2 LABELLING OF OLIGONUCLEOTIDE PROBES WITH DIGOXIGENIN

Digoxigenin kits (Boehringer Mannheim) were used to label oligonucleotides. This was done using the following components:

4 µl Tailing buffer

4 µl 25mM cobalt chloride

1 µl of oligonucleotide (0.1mM)

1 µl DIG-dUTP

9 µl milli-Q water

Finally 1 µl of terminal transferase was added and the mixture incubated at 37°C. After 15 minutes the tube was put on ice and made up to a volume of 100 µl with milli-Q water. 25 µl of this was used for probing the filters in one Hybaid bottle.

2.3.3 HYBRIDISATION OF DIG-LABELLED PROBE AND STRINGENCY WASHING

(Originally all probes were standardised using the TMAC hybridisation solution. However, this is a toxic and expensive agent, so in some methods SSC hybridisation solution was used instead)

This procedure was performed in Hybaid bottles rotating in a Hybaid rotisserie oven at the temperatures mentioned. Up to 2 blotted and fixed nylon filters were placed in each Hybaid bottle so that the side with the single stranded DNA was facing internally and the filters were non-overlapping. The filters were blocked for 20 minutes using 10ml of blocking reagent at room temperature. This solution was then discarded and the filters prepared for hybridisation by adding 10ml of hybridisation solution and incubating them at 5°C below the stringency temperature for 20 minutes. 25 µl of digoxigenin-labelled

probe was then added to each tube and hybridisation performed for at least 1 hour at the same temperature as the pre-hybridisation. The filters were rinsed for 20 minutes at room temperature with Wash Buffer. The stringency wash was then performed by incubating the filters with 10ml of TMAC hybridisation solution (TMAC method) or 10ml of 0.5 x Wash buffer (SSC method) at the stringency temperature for 15 minutes in order to leave just specifically bound probe attached to the DNA on the filter. (An estimation of the stringency temperature was made using the formula T_m (°C) = 4(G + C) + 2(A + T).

2.3.4 DIG DETECTION

The filters were rinsed with Buffer 1, then blocked with 10ml of Buffer 2 at room temperature. After 20 minutes 1µl of anti-dig-AP was added to the buffer 2 and incubated for a further 30 minutes. The solution was then discarded and the filters washed with Washing Buffer at room temperature for 20 minutes. The filters were rinsed in buffer 3 for 3 minutes, drained well, and then incubated with 5ml of CSPD solution for 3, 5 or 8 minutes, depending on how many times the solution had been used previously. The filters were then wrapped in saran wrap, incubated at 37°C for 15 minutes, and exposed to X-Ray film for an average of 15 minutes. A decision was made as to whether the stringency wash needed repeating at a higher temperature (in which case the method was restarted at the stringency wash step), or the filters needed re-probing at a lower temperature (in which case the method was started from the beginning).

2.3.5 STRIPPING DIG-LABELLED OLIGONUCLEOTIDES FROM HYBRIDISED NYLON FILTERS

If the filters were scorable and an alternative allele was to be probed for, the filters

had to be stripped before reprobing. To start with the filters were incubated with 10ml of Stripping Buffer 1 at 65°C for 30 minutes. The solution was discarded and replaced with 10ml of Stripping Buffer 2 for 20 minutes at room temperature. Finally the filters were incubated with 10ml of Stripping Buffer 3 at 37°C for 30 minutes.

2.3.6 STATISTICAL METHODS USED FOR CASE-CONTROL STUDIES

SPSS was used to analyse the data from the case control studies. The Statcalc programme in EPI INFO was used for 3 x 2 (2 degrees of freedom {d.f.}) and 2x2 (1 d.f.) χ^2 values, and calculation of odds ratios. Logistic regression was used to control for the confounding effects of ethnic and regional groups, age and sex in the Mali study using SPSS.

2.4 STOCK SOLUTIONS

2.4.1 SOLUTIONS USED FOR DNA EXTRACTION

Nucleon Reagent A -10mM Tris-HCl, 320mM Sucrose, 5mM $MgCl_2$, 1% Triton-X, adjusted to pH8.0 using 40% NaOH and autoclaved.

Nucleon Reagent B - 400mM Tris-HCl, 60mM EDTA, 150mM NaCl, 1% SDS (added after autoclaving), adjusted to pH8.0 using 40% NaOH and autoclaved.

10^{-4} x TE buffer - 10mM Tris-HCl, 0.1mM EDTA, adjusted to pH8.0 using 40% NaOH and autoclaved.

5M sodium perchlorate - supplied with kit

Nucleon silica suspension - supplied with kit

2.4.2 GENERAL STOCK SOLUTIONS

500Mm EDTA - 186.1g Na_2EDTA, made to pH 8.0 with NaOH

10% SDS - 100g sodium lauryl sulphate

20 x SSC -175.3g NaCl, 88.2g Na citrate, made to pH 7.0 with NaOH

20 x SSPE -175.3g NaCl, 31.2g $NaH_2PO_4.H_2O$, 7.4g EDTA made to pH 7.4 with NaOH

50 x TAE buffer - 242g Tris base, 57.1ml glacial acetic acid, 37.2g $Na_2EDTA.2H_2O$

10 x TBE buffer -108g Tris base, 55g boric acid, 40ml 0.5M EDTA, pH 8.0

TE buffer -10mM Tris.Cl, 1mM EDTA made to pH 8.0 with NaOH

2.4.3 PCR BUFFERS

10 x KCl Buffer - 100mM Tris HCl pH8.3, 15mM $MgCl_2$, 250mM KCl

10 x Ammonium Buffer - 160mM $(NH_4)_2SO_4$, 670mM TrisHCl (pH 8.8), 0.1% Tween20

2.4.4 REAGENTS USED FOR SEQUENCE SPECIFIC OLIGONUCLEOTIDE PROBING

DNA denaturing mix (100 µl/10µl of DNA)- 6µl 0.5M EDTA (pH 8.0), 86µl TE buffer (pH 8.0), 8µl 6M NaOH (fresh)

Blocking Solution - 4 x SSPE, 1% Blocking reagent stock (supplied by Boehringer Mannheim), 0.1% lauryl sarcosine

TMAC Hybridisation solution - 3M TMAC, 50mM Tris (pH 8.0), 0.1% SDS, 2mM EDTA

SSC Hybridisation solution- 5x SSC, 1% blocking reagent, 0.1% lauryl sarcosine, 0.02% SDS

10x Buffer 1- 1.5M NaCl, 1M maleic acid, made to pH 7.5 with NaOH (approx. 80g)

Wash buffer - 2 x SSPE, 0.1% SDS

Washing buffer -1 x Buffer 1, 0.3% Tween20

Buffer 2 -1 x Buffer 1, 1% Blocking reagent stock (kept at 4°C)

Buffer 3 - 100mM Tris (pH 9.0), 100mM NaCl, 20mM $MgCl_2$

CSPD - (supplied by Boehringer Mannheim) 1% in Buffer 3 (can be used 3 times within 48hrs. provided stored in light-proof container at 4°C)

Stripping buffer1 - 50mM EDTA (pH 8.0), 2 x SSC

Stripping buffer 2 -2 x SSC, 0.1% SDS

Stripping buffer 3 - 0.2M NaOH, 0.1% SDS

2.5 SUMMARY OF THE METHOD USED FOR GENOME SCREENING

2.5.1 INTRODUCTION

Now that numerous microsatellite marker loci are available (Dib et al. 1996), and there is the technology to accurately size them, whole genome linkage mapping of complex diseases has become possible. Sets of up to 8 markers that have non overlapping allele sizes can be run in the same gel lane together with an internal size standard. By labelling primers with one of three fluorescent markers that emit different wavelengths (Ziegle et al. 1992), 3 markers with overlapping size ranges can be run in the same lane together with a size marker labelled with a fourth dye, and each can be distinguished (fig 2.1). As a result, up to 24 microsatellite markers from one individual can be run in one lane (see appendix for marker sets) (Schwengel et al. 1994). This means that, in theory, 96 nuclear families could be screened for 288 markers by loading just 144 polyacrylamide gels.

The ABI machines have a laser that continuously scans the gel detects the fluorescent labels as they pass through the gel under the influence of the electric current. Sophisticated software can be used to calculate the allele sizes by comparing them with the size standard, and the table produced can be analysed by any of the numerous linkage

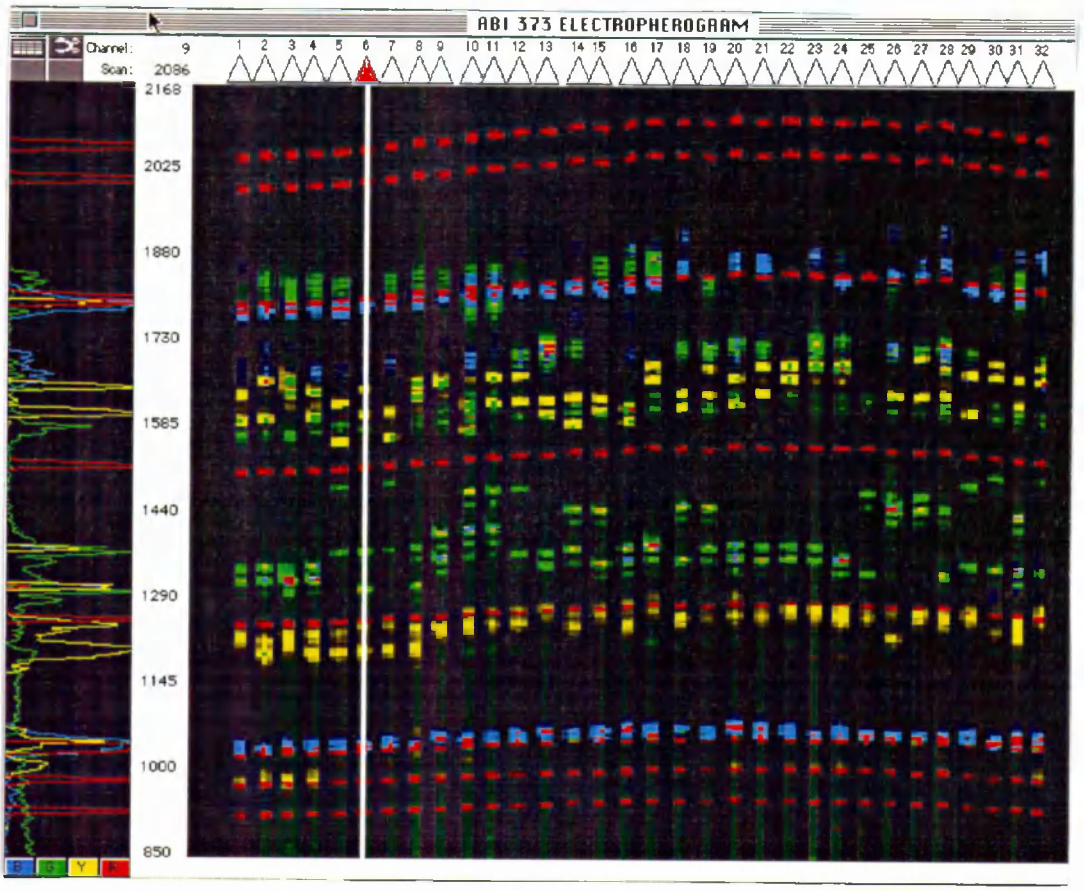

Fig. 2.1 Gel picture produced by Genescan Analysis™.

Each vertical line represents one lane and contains the pooled products of microsatellite pcr reactions from one set of markers in one individual. The red marks are produced by the Tamra labelled size standard. The yellow, green and blue marks are produced from the Hex, Tet and Fam labelled primers that have been incorporated into the products during pcr amplification.

programs now available. With the possibilities of multiplexing PCRs, robotic devices, and even better software, the whole process is likely to become even faster in the future.

2.5.2 LARGE SCALE DNA AMPLIFICATION

92 families with adequate amounts of DNA, and in whom the parents were as distantly related as possible, were designated to be used in the first round of the genome screen. A further 96 families were chosen for the second round. The DNA was diluted to a concentration of between 170 – 230 ng/ml using 10^{-4} TE buffer, and then further diluted to 10ng/ml with milli-Q water. This stock was placed in four 96-well deep-well plates (Beckmann), with the samples arranged in a predetermined fashion. This allowed easy dispensing of the DNA into polypropylene plates (Costar) for PCR using multi-channel, multi-dispensing pipettes.

PCR optimisations of the microsatellite markers belonging to one set were performed using spare DNA samples, by the standard techniques described above. A control sample containing water rather than DNA was always run, and the size of the products checked against a marker (ΦX 174). Once optimised, stock sufficient for all 4 plates was made up, Taq being added last. Using multichannel pipettes, the stock could be dispensed rapidly into ready prepared 96 well polypropylene plates (Costar) containing the correct amount of DNA and mineral oil. The plates were centrifuged briefly at 1000rpm before the PCR was performed. Four to six wells from each plate were checked for success of the reaction by running the product on agarose gels as described above. The basic 15μl reaction mix was as follows:

5 μl of DNA at 10ng/μl

1 x KCL buffer

1 – 4mM $MgCl_2$

200μM dNTPs

4 μM of each primer

0.2 units of Taq (initially Amplitaq, but changed to TAQ gold in January 1997)

1 drop of mineral oil

PCR cycles used in the genome screen are shown in Table 2.6 and conditions for the different primers are summarised in appendix 2.

2.5.3 POOLING OF PCR PRODUCTS

Once all the PCRs from one set had been successfully performed on all the families, the products were pooled as follows. 5 μl of the products of all the markers labelled with one particular dye were aliquoted into a fresh 96-well plate. In this way a pool of Hex, Tet and Fam was created corresponding to each of the four 96-well deep-well plates. Pools of the pools were then made so that the ratio of product was 4 Hex : 2 Fam : 1 Tet. An equal volume of milli-Q water was added to each pool, and the trays were centrifuged briefly at 1000 rpm.

2.5.4 PREPARATION OF THE SAMPLES FOR GEL LOADING

A 1: 5 solution of loading dye: formamide was made up and 3.2 μl of the resulting solution was added to 0.8 μl of the Tamra labelled marker dye, for every sample to be loaded. (The Tamra marker had fragments of the following sizes: 50, 75, 100, 139, 150,

Standard	Long (L)	Extra Long (XL)
94°C x 14 mins	94°C x 14 mins	94°C x 14 mins
95°C x 1 s } 49-61°C x 20 s}x 38 72°C x 5 s }	95°C x 1 s } 49-61°C x 20 s}x 38 72°C x 10 s }	95°C x 1 s } 49-61°C x 20 s}x 40 72°C x 10 s }
72°C x 1 min	72°C x 1 min	72°C x 1 min

Table 2.6 PCR programmes used in the genome screen (performed on MJ thermal cycler)

(Conditions are given in the appendix)

160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 base pairs). 4μl of this solution was added to 3 μl of each pool of pool sample in a clean Costar plate. Just prior to loading the gel, these samples were denatured at 95°C for 2 minutes, then put on ice. 3.5 μl of each sample was loaded into the wells of the acrylamide gel once it had been fully prepared (see below).

2.5.5 SETTING UP POLYACRYLAMIDE GELS

6% acrylamide/7M urea (Severn Biotech) 36 well gels were prepared for use by the core facility. They were allowed to set for at least 2 hours before use. The glass plates were then thoroughly cleaned under distilled tap water and dried clean so that there was no smearing. The comb was carefully removed and the gel placed in position in the 373A ABI sequencer. A plate-check was performed to ensure that there were no significant peaks on the trace that would subsequently interfere with the output. The voltage was adjusted so that the y co-ordinate of the trace lay between 800 – 1000. The wells were flushed with TBE buffer, the gel loaded in a predetermined order, and the run begun. Runs were performed using "Filter set B" for an average of 5 hours, or until the peak of the Tamra marker dye just larger than the largest expected allele size, had passed the laser.

2.5.6 GENESCAN ANALYSIS

The resulting gel files were transferred to Macintosh computers and analysed using Genescan Analysis™ as per instruction booklet. Default sample sheets (based upon the layout of the deep-well 96 well DNA plates) were entered onto the computer and saved. The gel file was then opened, the lanes tracked manually and the appropriate

ABI gel matrix and sample sheet imported. The Local Southern method was used for size-calling. Analysis was then initiated having defined red as the size standard colour and having entered the fragment sizes corresponding to the red peaks. Sample files were produced and saved.

2.5.7 GENOTYPING

Using the Genotyper™ software, templates were constructed for each marker set. Data concerning the marker name, expected size range, and fluorescent label were incorporated, and the templates saved. A macro was constructed so that the two highest peaks would be labelled, a table of the results produced, and the table rewritten after corrections had been made by the person checking the analysis.

Once a template had been produced, sample files from Genescan Analysis™ were imported and analysis proceeded. Genotyper™ works by correlating the fluorescent intensity of the labelled fragment with the size information and marker loci information. Because the allele calling by Genotyper™ is not 100% reliable, all results were checked manually. This also allowed the detection of cases of non-parentage. Allele size data were exported and stored as Excel files.

2.5.8 PREPARATION FOR FURTHER ANALYSIS OF DATA

We used the MRC set of microsatellite markers used by Todd et al. in their screen for type 1 diabetes genes, plus a number of gap fillers and replacement markers, which took the total to 293 markers (Todd et al. 1990). The mean density of the markers was 11.7cM. Initially the markers were divided into 14 sets, but because of marker failures and gap fillers this was expanded to 20 sets. One primer for each microsatellite was

labelled with one of the following fluorescent dyes: Fam (blue), Hex (yellow) or Tet (green) (see appendix for details). Products were pooled, a Tamra labelled size standard added, and aliquots of this were loaded into 36 well polyacrylamide gels in 373A Applied Biosystems Sequencers.

A laser scanned the gel during electropheresis, picking up the different fluorescent signals from the DNA fragments (fig.2.1). This method can distinguish fragments within 1 base pair size difference. The gels were analysed using Genescan Analysis™ and Genotyper™ to produce peaks representing the different alleles (fig.2.2). Although polymerase slippage occurs in this method, resulting in stutter bands, the stutter peaks are not as tall as the true peaks, and interpretation of true peaks is generally straightforward. As a result, the problems that used to be encountered in interpreting autoradiographs with multiple bands have been overcome. Despite this, the labelling of peaks by the computer was always confirmed manually, as was inheritance. Global binning of alleles was performed using the GAS™ programme. Statistical sib pair analysis was performed using GAS™ initially, but then Sibpair™ and Mapmaker Sib™, as they were found to be more reliable programs (Young 1995) and (Davis and Weeks 1997).

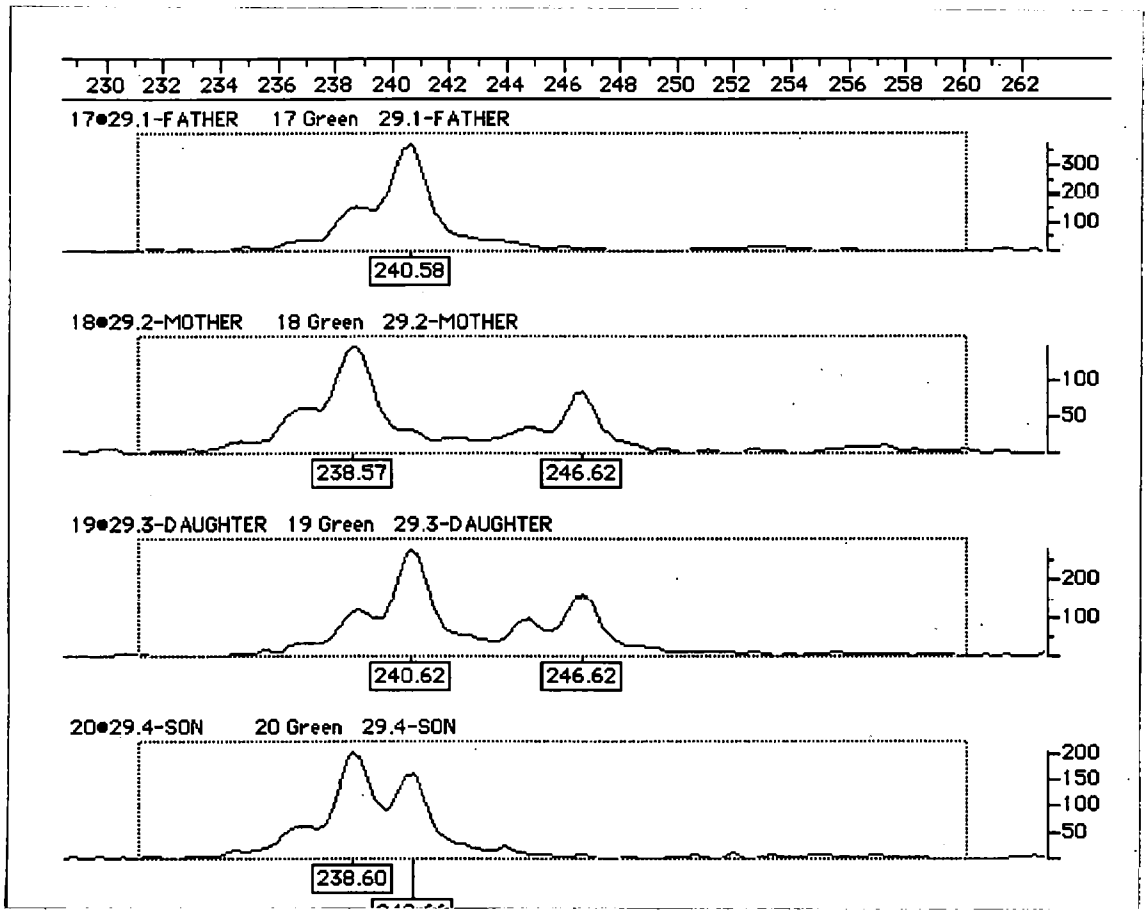

Fig. 2.2 Allele peaks produced by Genotyper™ for one microsatellite marker on one nuclear family. Note that the father is homozygous, so it is impossible to know whether the children are IBD or just IBS for the 240 allele

CHAPTER 3

3 SAMPLE COLLECTION AND CHARACTERISTICS

3.1 SAMPLE COLLECTION

Samples for this thesis came from a number of different sources. In this chapter I discuss some of the details behind the sample collection. Table 3.1 summarises the origins of the specimens used in different studies.

3.1.1 SAMPLE COLLECTION IN TAMIL NADU, SOUTH INDIA

India is the country with over half the world's leprosy, with an estimated 680,000 cases in 1997 (WHO 1997). There are some excellent leprosy control programmes in place with experienced staff surveying large populations. We collaborated with two such programmes in Tamil Nadu, South India, with the help of Professor R.M. Pitchappan from Madurai Kamaraj University. The leprosy projects were in Kumbakonam and in Sakthi Nagar (fig 3.1).

KUMBAKONAM

Kumbakonam is a small market town famed for its numerous temples. Over 90% of the population are rural, their main occupations being farming, silk weaving and labouring. Dr. T. Sitaraman and other members of the Lion's Club established the leprosy project in Kumbakonam in 1981. The aim of the project has been to detect new cases of leposy at an early stage, and to monitor the prevalence and incidence of the disease since the introduction of multidrug therapy.

Place	Country	Sib-Pairs +parents	Additional Cases	Controls	Collected and extracted by:
Kumbakonam and Sakthi Nagar, Tamil Nadu	India	242	132	201	S.Me & K.B.
Vishakapatnam	India	100	0	0	S.G. & team (minimal help from S.Me)
Bamako	Mali, West Africa	0	279	210	S.Mu & G.W (Co and Ex) S.Me (Ex)
Calcutta	India	0	227	165	S.R.

Table 3.1 Details of samples collected for genome screen and case control studies described in this thesis

K.B. =K.Balakrishnan S.G.= Satish Ghei S.Me = Sarah Meisner

S.Mu = Stuart Mucklow S.R. = Suschismita Roy G.W. = Giles Warner

Co = collected by Ex = extracted by

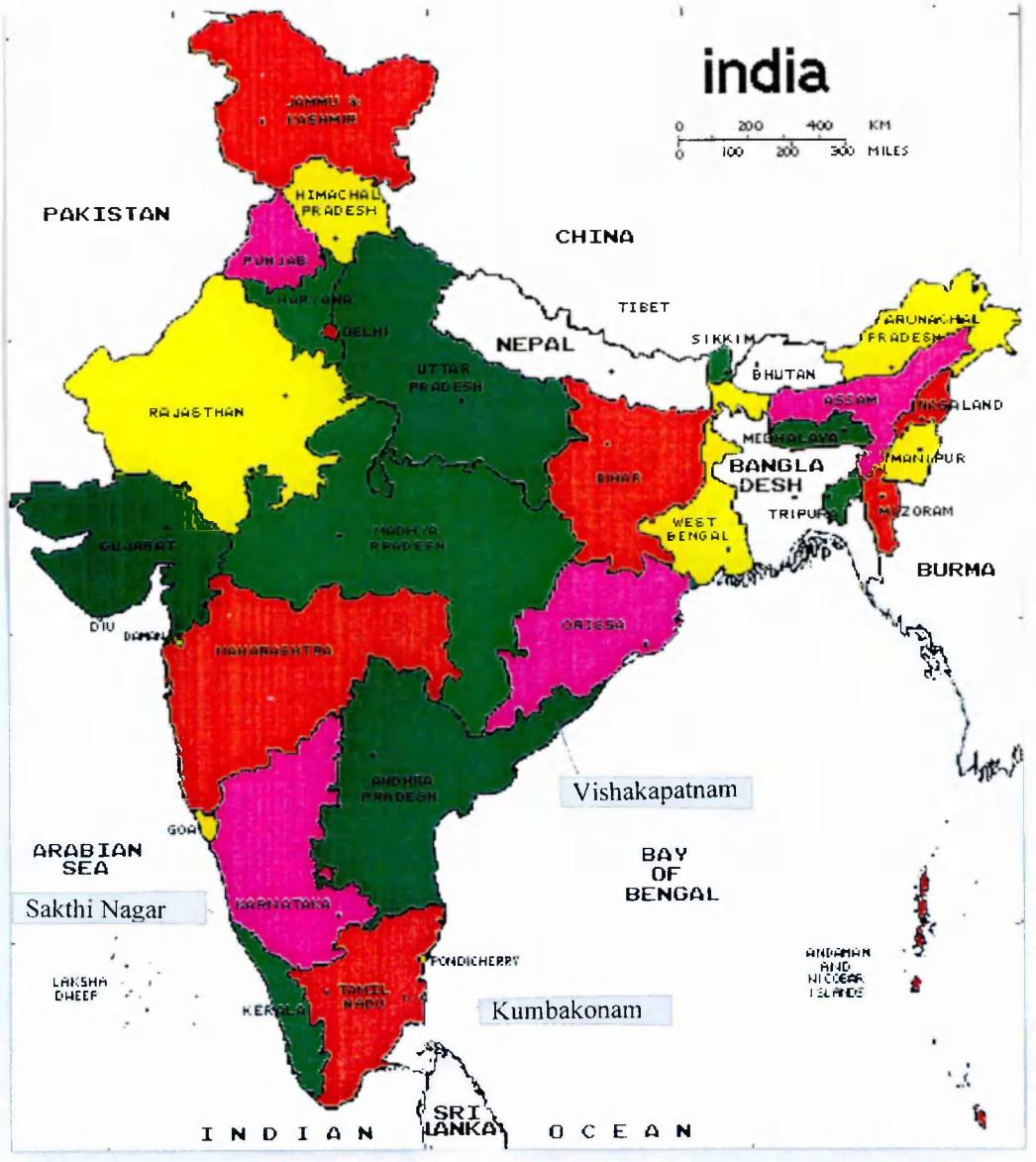

Fig. 3.1 Map of India showing Kumbakonam, Sakthi Nagar and Vishakapatnam

The headquarters are at the Hindu Mission Hospital in the town, but the 53 field workers go out to the villages and cover a population of more than 1.2 million in an area of 1892 square kilometres. There are three main administrative divisions each with a non-medical supervisor (NMS). The areas are divided into between 12 and 17 subdivisions, a field worker with a basic training in the diagnosis of leprosy being allocated to each subdivision (Sitaraman 1997). All school children are examined annually and the remainder of the population at least once every four years.

Whenever a field worker identifies a suspected case of leprosy, their non-medical supervisor (NMS) reviews the individual and their immediate family. If the NMS agrees with the diagnosis then any affected individuals are referred to the doctors, who hold clinics at local health centres and in Kumbakonam. If the NMS agrees with the diagnosis, the patient is referred to one of the mobile clinics where a doctor with specific training in leprosy makes a final decision on the diagnosis. All cases confirmed by the doctors are treated as per WHO regime, 6 months of Rifampicin and Dapsone for PB cases, and 2 years of Rifampicin, Clofazimine and Dapsone for MB cases.

There is no monetary incentive for staff to diagnose cases. Split skin smears are taken on all cases and a random selection of slides is checked annually at the Schifflien Leprosy Research Centre at Karigiri and to GREMALTES in Madras. W.H.O. consultants visit once every four months. Patients are followed up during treatment and are referred to the Sacred Heart Leprosy Hospital in Kumbakonam if there are any complications. In1997, 532,359 individuals were screened for leprosy by the Hindu Mission Hospital staff. 621 of those screened had leprosy (i.e. an annual rate of 1.17/1000 population

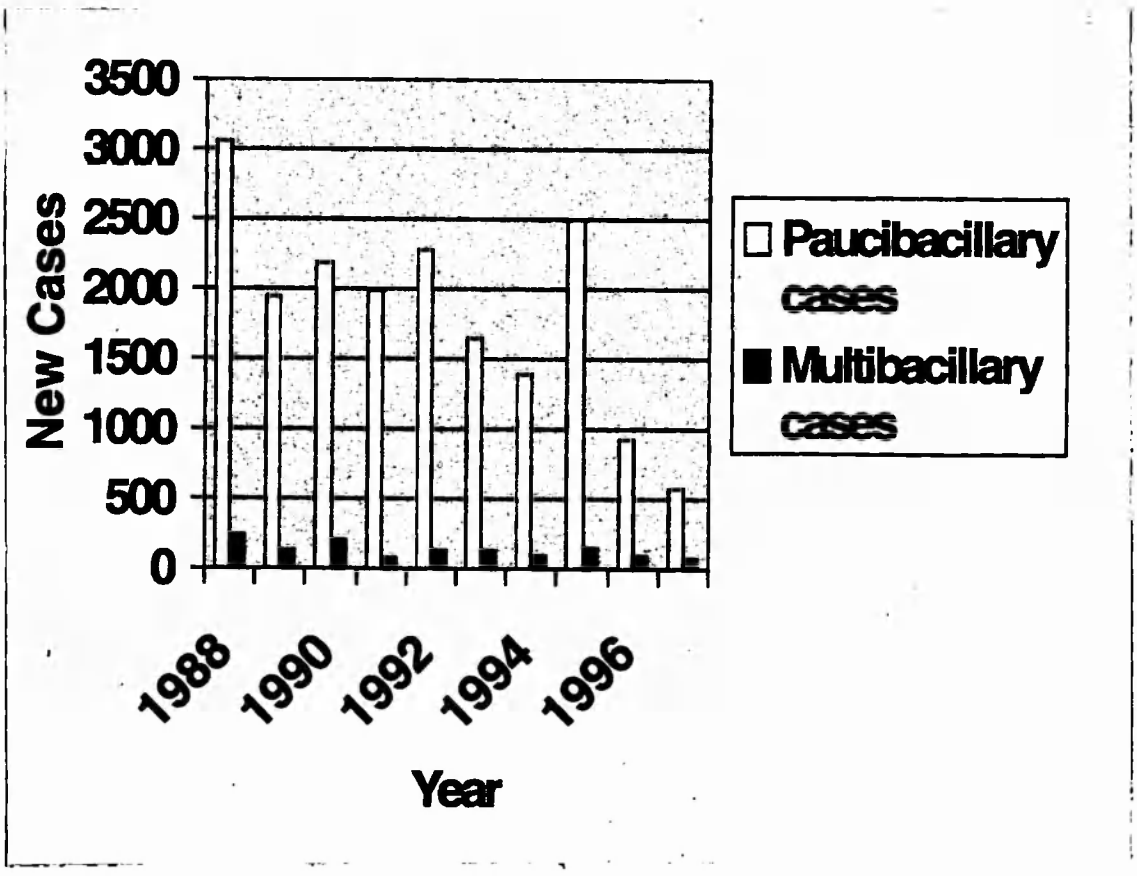

Fig. 3.2 A Bar Chart of the number of newly diagnosed cases per year in Kumbakonam since 1988

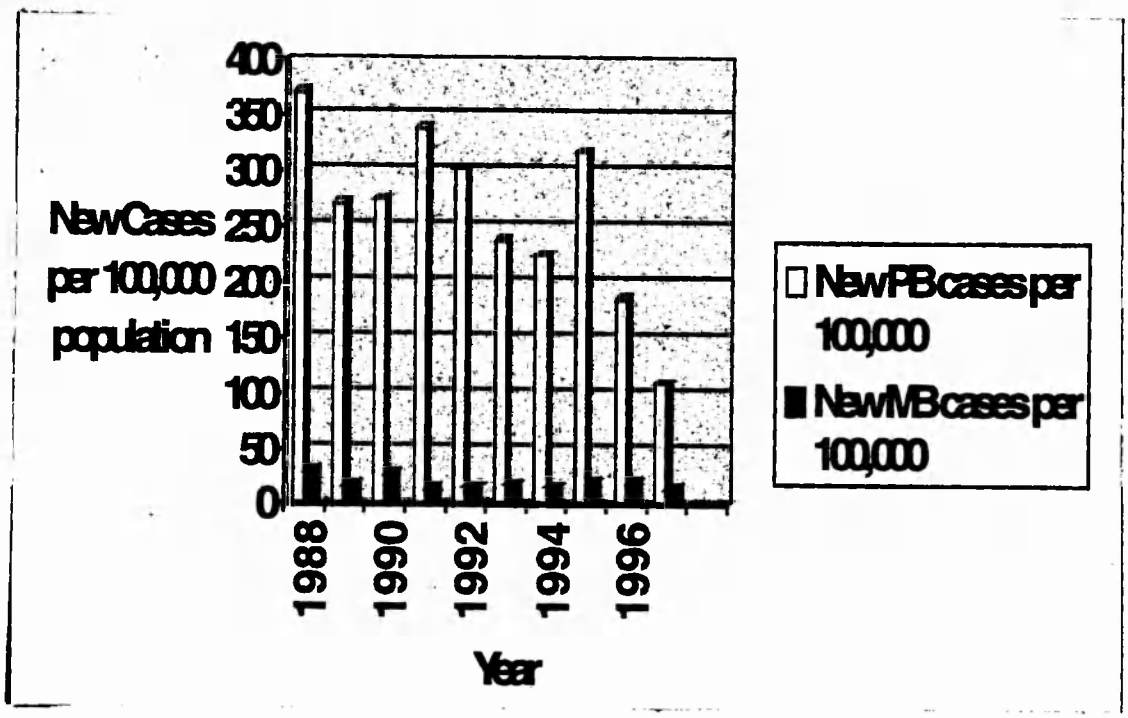

Fig. 3.3 A Bar Chart of the number of newly diagnosed cases per 100,000 of the population examined per year in Kumbakonam since 1988

screened).88.7% of these cases were paucibacillary. 63.6% were male. Only 1 of the 70multibacillary cases occurred in an individual under 15 years of age, whereas 71% of the paucibacillary cases were in this age group. In addition to the new patients there are 6423 patients currently under surveillance. Figures 3.2 and 3.3 show the distribution of new cases and new cases per 100,000 of the population in the surveillance area from 1988 to 1997.

SAKTHI NAGAR

Sakthi Nagar is a sugar factory in a rural area of Tamil Nadu about 20 miles from Erode. There is no town as such. The local population pursue similar professions to those in Kumbakonam, only a minority actually being directly employed by the factory. The factory managers established a hospital, and the Leprosy Project at Sakthi Nagar is an offshoot of this. Dr. N.K. Shanker Narayan currently runs it. The project is arranged in a similar way to that in Kumbakonam, with field-workers, non-medical supervisors and doctors. I have been unable to get up to date information on the current prevalence and incidence of leprosy in the area.

SAMPLE COLLECTION IN TAMIL NADU

I went on 2 field trips to India in order to collect samples for the genome screen. The first trip took place from July to October 1995, the second from October 1996 to January 1997. Blood was collected from a total of 220 families consisting of 2 parents and at least 2 siblings, both of whom had a previous diagnosis of leprosy. There are also 22 incomplete families. All families were from Tamil Nadu in South India, 182 of the families being from the Kumbakonam region, the remainder from Sakthi Nagar.

In addition to the families, I collected blood from a total of 201 controls matched for caste and, as frequently as possible, for area. The majority of the controls were neighbours of the families and none of them had any first-degree relatives with leprosy. Most were over 20 years of age. Finally, I collected blood from 100 patients with a past history of erythema nodosum leprosum (ENL), 44 lepromatous leprosy patients who had had no episodes of ENL over at least two years (referred to as "LL controls" in this thesis), and 16 patients who had suffered type 1 leprosy reactions. All of these patients were under the care of Dr. S. Thomson at the Sacred Heart Leprosy Mission at Kumbakonam. This hospital specialises in the care of leprosy patients from as far as 200 miles away including any complicated cases detected by the Hindu Mission Hospital. Details of the families collected are shown in the tables 3.2 to 3.5.

In the vast majority of families blood was sampled from a nuclear family comprising two affected siblings and their parents. In 11 families there were three affected siblings, and 1 family had 4 affected children (table 3.2). This latter family was the only one in which the individuals were not fully Tamil, the mother originating from Malaysia. Most of the siblings had been affected by tuberculoid leprosy, but in 13 families, one sibling had suffered from lepromatous leprosy. There were no families in which both siblings had had lepromatous leprosy (table 3.3). In 61 families one or other parent had been affected by leprosy at some stage, whilst both parents had been infected in 13 families (table 3.4). There is a large amount of consanguinuity in South India, cousin and uncle/ niece marriages being encouraged rather than stigmatised. Although 57% of the parents claimed to be unrelated, 20% of parents were first cousins and 7% uncle/ niece marriages (table 3.5).

Sib pairs in family	Incomplete family/insuff-icient DNA	2 siblings	3 siblings	4 siblings
No. of Families	33	201	11	1

Table 3.2 Breakdown of leprosy families collected from Tamil Nadu for sib-pair study

Case mix of families	Both sibs paucibacillary leprosy	1 sib paucibacillary 1 sib multibacillary	Both sibs multibacillary
No. of Families	221	13	0

Table 3.3 Breakdown of leprosy type observed in leprosy affected sib-pairs from Tamil Nadu (in families with just 2 affected siblings)

Parents affected	Neither parent affected (or information on 1 parent only)	1 parent affected	Both parents affected
No. of families	172 (70%)	61 (25%) (26 mothers, 35 fathers)	13 (5%)

Table 3.4 Leprosy cases in parents of families with 2 or more affected siblings from Tamil Nadu

Relationship of parents	Percentage of Families
Unrelated	57%
Distantly related	11%
First cousins	20%
Uncle/Niece marriage	7%
Unknown	5%

Table 3.5 Relationship of parents in families collected from Tamil Nadu for genome screen

SAMPLES FOR VISHAKAPATNAM

100 sets of affected sib-pairs and their parents were collected by Dr. Satish Ghei and his team from the Japanese Leprosy Mission Agra (JALMA). The samples were collected in Vishakapatnam in Andhra Pradesh, South India. JALMA has strong links with GREMALTES, a large leprosy screening programme similar to those in Tamil Nadu. Vishakapatnam is a large port town, people living in relatively poor urban conditions, but not slums.

CALCUTTA LEPROSY SAMPLES

Dr. Suschismita Roy collected these samples between 1992-1995 as part of her PhD at Cambridge. Cases and controls were recruited from the Leprosy clinic at the School of Tropical Medicine in Calcutta. The diagnosis was made by experienced physicians and reviewed by a panel of 3 leprologists following further investigations. Controls were blood donors from the Swasti Blood Bank in Calcutta. There were 121 patients with lepromatous leprosy (median age 33 years) and 107 with tuberculoid leprosy (median age 30 years) plus 160 controls (median age 34 years). There were 6 women in the tuberculoid leprosy group and 10 in the lepromatous leprosy group, none amongst the controls. Cases and controls were well matched for ethnic group and caste.

3.1.2 AFRICAN LEPROSY SAMPLES

Africa is the continent with the second largest leprosy problem after Asia with an estimated 140,000 cases in 1997. Mali, in West Africa, had 1581 new and 3248 registered cases of leprosy in 1997 (WHO 1997) (see Fig.3.4). The Institut Marchoux is the national leprology centre for Mali and also the leprosy reference centre. In addition to leprosy the

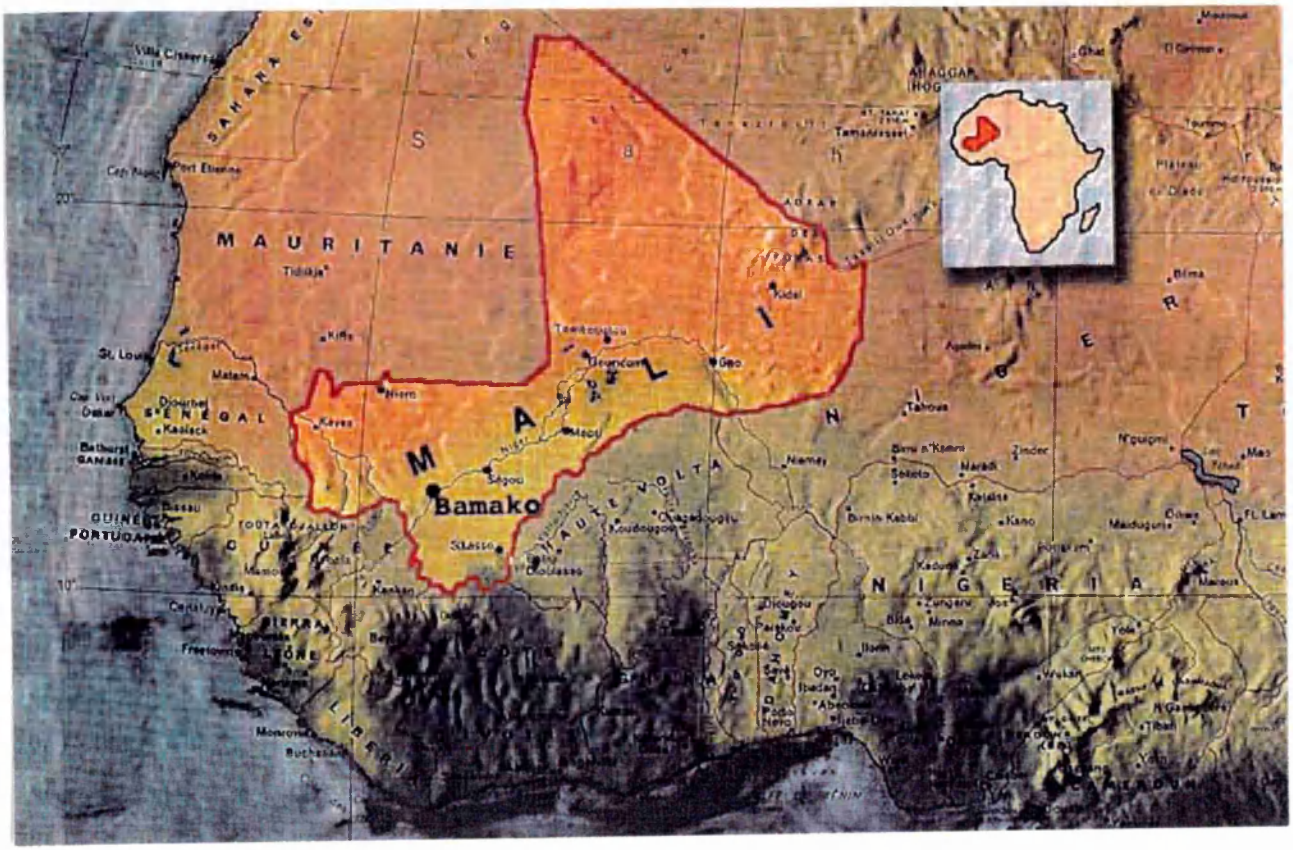

Fig.3.4 Map of Mali

Institut also specialises in dermatology. It has a busy outpatient department as well as a 30 bedded leprosy ward.

Samples were collected by Stuart Mucklow and Giles Warner with the co-operation of the Institut Marchoux in Mali. The cases recruited were out-patients, in-patients and ex-patients from the leprology unit. Details were taken of age, sex, ethnic group, form and type of leprosy, stage of treatment and BCG status. Controls were recruited from those attending the phlebotomy department, staff members, unaffected family members of leprosy cases and non-leprosy patients attending the general medical or infectious diseases out-patient clinics at the Institut Marchoux. All controls were questioned and examined to exclude the possibility of them having leprosy.

The cases were of all clinical types, about 2/3 being multibacillary, 3/4 of whom had polar lepromatous leprosy (tables 3.6 and 3.7). There was a higher proportion of men amongst the cases than the controls, and controls had a lower mean age than the cases. Just over 50% of the cases collected had experienced leprosy reactions at some stage, the large proportion reflecting the fact that samples were collected from a tertiary referral centre(Table 3.8). Over 70% of the reactions were type 2 in nature, the excess number mirroring the excess number of lepromatous cases. 55% of people with type 1 reactions had multibacillary leprosy, 45% paucibacillary again reflecting the skew in lepromatous cases collected.

Over 44% of the participants originated from around Bamako (Table 3.9). Cases were significantly under-represented amongst this group ($p<0.001$), which is not surprising given the nature of referrals to this hospital, some leprosy patients travelling

great distances for expert advice. The reverse pattern is seen in people from administrative districts V and VII ($p<0.001$). The four largest ethnic groups were the Bambara (40%), the Peulh/Foulah (20%), the Malinke (14%) and the Sarakoli (10%) (Table 3.10). There were an excess of cases compared to controls amongst the Bambara and the Peulh/Foulah ($p=0.005$), whereas in the other two groups the differences were less marked and were in the opposite direction.

Leprosy Type	No. of females	No. of males	Total No. of samples	Mean Age(years)
MB	53(28.6% of MB cases)	132(71.4% of MB cases)	185(66.3% of cases)	45.7 (s.d.14.4)
PB	31(33.0% of PB cases)	63(67.0% of PB cases)	94(33.7% of cases)	44.9 (s.d.14.9)
Total No. of Cases (MB+PB)	84(30.1% of cases)	195(69.9% of cases)	279	45.4 (s.d.14.6)
Controls	133(63.3% of controls)	77(36.7% of controls)	210	32.5 (s.d.13.0)

Table 3.6 Age, Sex and Case distribution of samples from Mali (MB = multibacillary, PB = paucibacillary leprosy cases)

Leprosy Type	No. of Cases
Indeterminate	2 (0.7%)
TT	46 (16.5%)
BT	49 (17.6%)
BB	5 (1.8%)
BL	46 (16.5%)
LL	131 (47.0%)

Table 3.7 Frequencies of different types of leprosy in cases from Mali

(TT = tuberculoid, BT = borderline tuberculoid, BB = borderline borderline, BL = borderline lepromatous, LL = lepromatous leprosy)

Reaction Type	No. with reaction	% MB	%PB
No reaction	130 (46.6%)	42.3	57.7
Type 1 reaction	42 (15.1%)	54.8	45.2
Type 2 reaction	107 (38.4%)	100	0

Table 3.8 Frequency of leprosy reactions in leprosy patients from Mali

(MB = multibacillary, PB = paucibacillary)

Region*	MB (% of total cases & controls in the region)	PB (% of total cases & controls in the region)	Total Cases (% of total)	Controls (% of total)	Total (% of total)
I	27 (71`%)	11 (29%)	38 (14%)	26 (12%)	64 (13.1%)
II(Bamako/ Koulikoro)	64 (69%)	29 (31%)	93 (33%)	123 (59%)	216(44.2%)
III	12 (57%)	9 (43%)	21 (8%)	15 (7%)	36 (7.4%)
IV	30 (75%)	10 (25%)	40 (14%)	23 (11%)	63 (12.9%)
V	28 (57%)	21 (43%)	49 (18%)	8 (4%)	57 (11.7%)
VI	11 (61%)	7 (39%)	18 (6%)	11 (5%)	29 (5.9%)
VII	2 (40%)	3 (60%)	5 (2%)	0	5 (1.0%)
VIII	0	0	0 (0%)	1 (1%)	1 (0.2%)
Other West African countries	11 (73%)	4 (27%)	15 (5%)	3 (1%)	18 (3.7%)
Total	185 (66%)	94 (34%)	279	210	489

Table 3.9 Regional distribution of cases and controls from Mali (MB =multibacillary, PB = paucibacillary)

* The numbers refer to the administrative subdivisions of Mali

Ethnic Group	MB (% cases for ethnic group)	PB (% cases for ethnic group)	Total no. of Cases (% of total cases for ethnic group)	Controls (% of total cases for ethnic group)	Total
Bambara	79 (67%)	39 (33%)	118 (43%)	71 (35%)	189 (40%)
Bobo	1	1	2	1	3
Bozo/Somono	5	1	6	3	9
Dogon	7	2	9	2	11
Forgeron	1	0	1	3	4
Kassouke	2	1	3	2	5
Kissie	0	0	0	1	1
Malinke	16 (59%)	11 (41%)	27 (10%)	37 (18%)	64 (14%)
Minianka	0	0	0	5	5
Moor	3	0	3	1	4
Ouolof	1	0	1	1	2
Peulh/Foulah	36 (59%)	25 (41%)	61 (22%)	35 (17%)	96 (20%)
Sarakole	18 (72%)	7 (28%)	25 (9%)	24 (12%)	49 (10%)
Senoufo	0	1	1	5	6
Song/rhai	9	4	13	9	22
Sonike	3	0	3	0	3
Touareg	0	0	0	1	1
Total	181 (66%)	92 (34%)	273	201	474

Table 3.10 Ethnic group distribution of cases and controls from Mali

(MB = multibacillary cases, PB = paucibacillary cases)

CHAPTER 4

4 GENOME SCREEN

4.1 INTRODUCTION

As discussed in chapter 1, it is clear that susceptibility to leprosy has a genetic component (Fine 1981) and the evidence from segregation analyses points towards the possibility of a major gene effect (Abel and Demenais 1988). There are two main approaches to finding genes in complex diseases: linkage studies and association studies. Association studies are discussed in section 1.8.1. In the linkage approach the entire genome is systematically screened for linkage of major susceptibility loci to regularly spaced markers. It is an expensive and labour intensive method, but provided the markers are suitably spaced it will identify major genes. It has been used successfully in a number of complex disorders where there are several genes that interact with the environment e.g. types 1 and 2 diabetes and asthma (Davies et al. 1994), (Daniels et al. 1996). Because families are needed, there can be problems when studying diseases that have a late onset as parents will be unavailable. Identity by state methods can overcome this problem by making a number of assumptions.

The chronicity of leprosy and the fact that it frequently affects relatively young individuals makes it an ideal disease to study by this method, especially compared to most other infectious diseases. In this chapter I will discuss the genome wide search approach that we used to search for leprosy susceptibility genes.

4.1.1 COMPLEX DISEASE

Any multifactorial disease that deviates from classical Mendelian rules is said to be complex. This may be because there are several genes controlling that particular phenotype, i.e. genetic heterogeneity, or there may be "phenocopies", where a phenotype is produced by environmental rather than genetic causes. Interactions with other genes and with the environment may result in a given genotype producing a number of different phenotypes. Penetrance of a genetic disease can vary for reasons not yet understood, or may be age or sex related as in the case of the BRCA1 gene (Newman et al. 1998). In some cases several mutations need to be inherited at a number of loci before a disease will manifest itself, i.e. there is polygenic inheritance as occurs in some forms of Hirschsprung's disease (Israel and Dunant 1998). Other complicating factors include a high frequency of disease causing alleles in the general population, imprinting, anticipation and mitochondrial inheritance (Lander and Shork 1994).

4.2 LINKAGE ANALYSIS

4.2.1 BACKGROUND TO LINKAGE ANAYSIS

The term "linkage" was first coined in the early years of this century. It refers to the tendency of genes to be inherited together as a result of their close location on the same chromosome, thus deviating from the predictions of Mendel's law of independent assortment. The percentage recombination between two loci is a measure of the degree of linkage and, over short distances, can be roughly correlated with physical distance between the loci. Linkage analysis involves proposing a model to explain the inheritance patterns observed in a pedigree. The objective of linkage analysis is to identify the map location of disease genes where the biochemical mechanism is unknown. Genes may be

detected despite genetic heterogeneity, incomplete penetrance, variable age of onset, and the presence of families with non-genetic causes. By identifying the gene and its molecular sequence, the biochemical mechanism may be determined, and cures for the disease established

Originally a paucity of markers hampered progress in genetic mapping, blood group antigens being one of the few polymorphic alleles that could be used. During the 1980's methods became available to locate genes responsible for simple Mendelian genetic traits (Botstein et al. 1980). With the advent of dense genetic maps (Dib et al. 1996), automated sequencers, advanced computer software, and even robots, the possibility of dissecting the genetics of complex multifactorial diseases is now a reality. Genome screening was first used successfully in animal models e.g. NOD mice, but since then numerous studies have been performed on polygenic human diseases, such as diabetes (Davies et al. 1994) and asthma (Daniels et al. 1996).

Linkage studies can either be parametric or non-parametric. In the former a mode of inheritance is postulated and the theory tested using lod scores (see section 4.2.5). When the mode of inheritance is known this method is statistically efficient, gives an idea of the location of the gene and allows both linkage detection and exclusion. Non-parametric methods are a more appropriate method for linkage detection in complex diseases where the mode of inheritance is unknown. Maximum likelihood interval mapping is used to analyse markers spaced at 10-20cM intervals on affected relative pairs (Hauser et al. 1996).

Factors that may disturb the usual inverse relationship between linkage disequilibrium and genetic distance include mutation, genetic drift, population admixture,

genetic selection and variance of the sample. In addition, recombination events are more frequent in telomeric regions than elsewhere on the chromosome, and in certain genes e.g. human immunoglobulin genes there is a high recombination rate in somatic events, but not in meiosis (Watkins et al. 1994). Gene localisation by linkage methods is limited to an approximately 1-2cM region since the number of meioses required to detect recombinants becomes very large as the distance becomes smaller.

Linkage disequilibrium is the non-random segregation of alleles linked to a locus. It relies on the fact that the closer two alleles are on a chromosome, the more likely they are to be inherited together. Descendants, who inherited a disease allele from an ancestor, are also likely to share the ancestral haplotype in the region of the disease locus. The size of this region depends upon the recombination rate and the age of the mutation. Linkage disequilibrium methods are useful in reducing the size of the region detected by linkage methods and have been used successfully in localising several genes e.g. the cystic fibrosis gene (Terwilliger 1995).

4.2.2 MICROSATELLITE MARKERS

Microsatellites are tandem repeats of nucleotides (most commonly (CA) repeats) that are interspersed throughout the genome, and probably arose due to polymerase slippage during evolution (Schlotterer and Tautz 1992). These markers are inherited in a Mendelian fashion and are polymorphic with a relatively low mutation rate (Dib et al. 1996). Botstein suggested that a map of 150 markers that were sufficiently polymorphic, with spacing of approximately 20cM, was adequate to detect linkage to any given locus (Botstein et al. 1980). One suggested screening method that could reduce the workload

and expense of a genome screen is to only screen those markers in GC rich regions of the genome (Antonarakis 1994).

Various groups have worked on the production of sets of microsatellite markers that can be run in one gel lane concurrently, thus reducing the number of gels that need loading (Schwengel et al. 1994). Using fluorescently labelled markers it is possible to run large numbers of products in the same gel lane without producing overlapping bands. Markers are chosen based on their polymorphic information content (PIC), map position, and whether they amplify well on PCR and produce clear bands on the Genotyper™ programme.

4.2.3 RISK OF DEVELOPING DISEASE TO RELATIVES OF AN AFFECTED INDIVIDUAL

The concept of λ_R, the risk to a relative, R, of an affected individual compared to the risk in the general population, is a useful measure of the genetic risk - the larger λ_R, the greater the genetic effect (Risch 1990a). In insulin dependent diabetes, λ_S, the risk to a sibling, S, compared to the population, is 15, whereas in cystic fibrosis it is 500 (Davies et al. 1994). Risch estimated the λ_S in tuberculoid leprosy to be 2.38 (Risch 1987). When genes are identified, the contribution of the gene to the λ_S can be calculated, so that an estimate of the number of major genes remaining can be made though the interaction of the genes may be additive or multiplicative (Cordell and Todd 1995).

4.2.4 IBD VS IBS

Any two copies of an allele A at a locus are said to be identical by state (IBS). However, they are only identical by descent (IBD) if they are both inherited from a common ancestor. Two alleles that are IBD are also IBS, but the converse does not

necessarily follow. It has been argued that provided a marker is sufficiently polymorphic, IBS status will approximate to IBD status (Weeks and Lange 1988).

4.2.5 LOD SCORES

The lod score is the "logarithm of the odds" ratio of having the observed result at a given recombination fraction, θ (where $0<\theta<0.5$), compared to the result expected if the null hypothesis were true. It represents a measure of the evidence for linkage versus the absence of linkage. By convention in classical parametric linkage studies, a lod score of more than 3 is taken as being highly suggestive of linkage, which corresponds to an odds ratio of 1000:1. There has been a great deal of discussion over the appropriate threshold that should be applied to genome screens. The main problem is that every case will differ in terms of the genetic model, the markers screened and their heterozygosity and informativeness (Lander and Kruglyak 1995). It has been suggested that simulation models be performed to calculate appropriate thresholds (Lander and Shork 1994).

To calculate the expected lod score one needs to know all the possible phenotypic constellations for that family type and their probability of occurrence. At a given recombination fraction, θ, the expected lod score is the weighted average of the $z_i(\theta)$ over all possible outcomes, where z_i is the probability of an affected sib-pair sharing i alleles identical by descent. The maximum lod score (MLS) is frequently quoted and represents the maximum score reached after varying the parameters, e.g. θ, allele frequencies, penetrance and phenotypic variance.

4.2.6 THE POSSIBLE SHARING TRIANGLE

When multiple calculations of the lod score are performed at different recombination fractions, implausible results may occur if the possible sharing triangle is not taken into

account. The following restrictions have been found to hold:

$$z_1 > 0$$

$$z_0 + z1 + z_2 = 1$$

$$z_1 = 2z_0$$

This leads to a triangle bound by $z_0 = 0$, $z_1 = 0.5$, and $z_1 = 2z_0$. When this method is applied, the MLS is the maximum lod score satisfying the above conditions (Holmans 1993).

4.2.7 EXCLUSION MAPPING

Exclusion mapping is a parametric method used to identify chromosomal regions that are unlikely to be linked to a disease. Lod scores are calculated as a function of θ and λ, such that areas of the genome producing a sufficiently low lod score can be excluded from containing the gene of interest at the specified value of λ. Assumptions have to be made concerning the mode of inheritance, the number of disease loci and/or the λ_s (Hauser et al. 1996). Lod scores of - 2 are generally taken to mean that that particular region has been excluded from linkage. This is probably the most powerful application of multilocus mapping (Edwards 1987).

4.3 AFFECTED SIB-PAIR METHOD

The affected sib-pair method is a non-parametric method of linkage analysis. It requires no prior assumptions as to the mode of inheritance, making it more robust than parametric methods. If the disease model is known correctly then parametric methods are more powerful, but if not, the sib-pair method is useful as it is less susceptible to modelling errors. It has greatest power when the disease is recessive. By focusing only on

affected children, problems of incomplete penetrance are circumvented and the need to control for environmental factors is eliminated. Complex diseases with genetic and aetiological heterogeneity and polygenic inheritance can be studied. An advantage over other types of linkage study is that nuclear families are generally easier to collect than large multiplex families. They are also more likely to represent the common form of the disease or trait.

In the affected sib-pair method one is testing for cosegregation of a marker allele with a disease-predisposing allele. Given traditional Mendelian rules of inheritance, 2 affected siblings have a 25% chance of sharing 2 alleles ibd at a particular locus, a 50% chance of sharing 1 allele ibd, and a 25% chance of sharing neither allele (see fig.4.1), thus the expected 2:1:0 sharing ratio is 1:2:1. An alternative way of looking at this is to

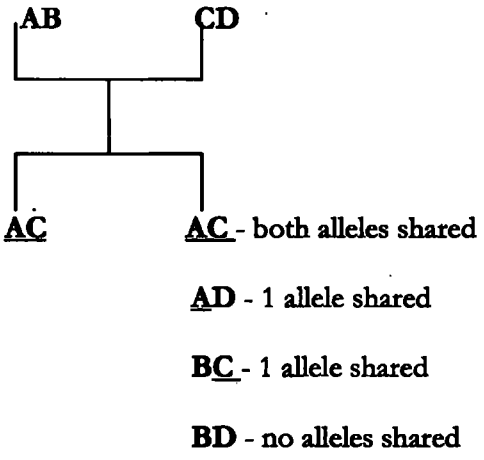

Fig.4.1 Sharing of alleles in siblings

consider the inheritance of alleles from one parent, i.e.1:0 sharing. Randomly selected siblings have a 50% chance of sharing an allele ibd. If siblings are selected that both suffer from the same genetically determined disease, there is a high probability that they will share the segment of chromosome that carries the disease susceptibility gene. As a

result of this, the percentage sharing of closely linked marker alleles will be greater than expected (Ott 1991).

Two or more siblings affected by a disease are typed for polymorphic markers such as microsatellite markers. If their parents are also typed, the interpretation of whether alleles are ibd or ibs is much easier and cases of non-paternity can be recognised and excluded. The percentage sharing of alleles identical by descent in a number of siblings is compared with that predicted by random segregation, the null hypothesis being that a given marker is unlinked to disease. The results are analysed by a Chi-squared test, comparing the number of shared alleles to the number that would be expected if the null hypothesis were true. A p-value of less than 0.05 is taken as an indicator of possible linkage. Lod scores can also be calculated. In the case of microsatellite markers, where perhaps 300 analyses are performed per family, a p-value of 0.05 is likely to occur 15 times by chance alone. One way of allowing for this is to divide the p-value by the number of tests to be performed or apply a correction, which allows for multiple testing e.g. the Bonferroni correction:

$$\alpha_i = 1-(1- \alpha_n)^{1/n}$$

where α_i is the significance level for each individual test and α_n is the overall significance level after n tests e.g. for a significance level of 0.05 over 300 tests, α_i is 1.7×10^{-4} (Weeks et al. 1997). This correction assumes that the tests are mutually independent. If too low a significance level is chosen then a type II error may occur. An alternative approach is to perform the genome screen in two stages. In the first stage all the markers at the chosen spacing are examined. Those markers that appear to be linked in the first round are re-examined in a second screen on a further set of families. Once linkages have been

confirmed, fine mapping can be performed or candidate genes in the region can be investigated using case control studies.

Unfortunately, not all meioses are informative even when both parents are available for typing. In the case of intercross parents who are both heterozygous for the same alleles, it is impossible to tell whether two children with the genotype AB share 0,1 or 2 alleles identical by descent. A similar scenario occurs in the case of a parent being homozygous at a locus (fig. 4.2). Multi-point methods of analysis, e.g. Mapmaker Sibs™, use information from nearby markers to estimate the sharing in cases such as these.

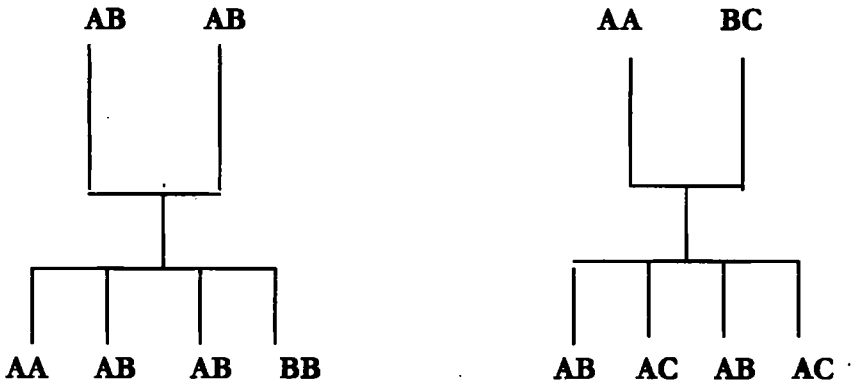

Fig. 4.2 Pedigrees demonstrating ambiguity seen during intercross matings and in cases of homozygosity in one of the parents

If the parents are unavailable, other family members can be used to increase the power. However, typing both parents is more informative than having one parent, which is more informative than having additional siblings. Risch found that the type of relative-pair best suited for a particular linkage study was dependent on the λ_R, θ, and

polymorphic information content (PIC) of the markers. For large values of λ_R and PIC, he found it worthwhile typing more distant relative pairs, whereas, in cases where the λ_R was small and the PIC less than 0.7, siblings were preferred (Risch 1990b). Hauser suggested using affected sibling pairs without their parents in initial screening. Although less information is available and assumptions are made in calculating maximum likelihoods, twice as many pairs can be typed for the same amount of lab work. This increase in information may lead to increases in the lod score of between 17-46% as well as decreasing the workload and expenditure of the genome screen (Hauser et al. 1996).

The power of a test is the probability of rejecting the null hypothesis when the null hypothesis is false. In linkage studies this depends upon the recombination fraction, the amount of data available, the spacing of the markers, and the λ value for that locus. In most cases the values for θ and λ will be unknown. Brown et al. suggested that 20cM intervals were a good compromise between power and workload in initial screens (Brown et al. 1994). On further screening higher density mapping can be performed on selected regions. Based on simulation studies, if 96 sib-pairs are investigated using microsatellite markers at 20 cM intervals, there is a 99.9% chance of detecting a lod score of 2.3 or more for a locus where the λ_S is 2.5 (Davies et al. 1994). Various formulae are available to calculate the number of families required to detect a significant linkage (Elandt Johnson 1971).

4.3.1 ADJUSTMENTS FOR FAMILIES WITH MULTIPLE AFFECTED SIBLINGS

In cases where there are more than 2 affected siblings from one family the number of sibling pairs cannot simply be calculated from {(n-1) + (n-2) + (n-3) +.....0}, as might

be expected, because the pairs are not mutually independent of one another (Hodge 1984). Knowledge of the allele sharing of two pairs of siblings in a family with 3 affected siblings, frequently allows deduction of the sharing in the third pair. In order to overcome this, a weighting function may be incorporated (Suarez and Van Eerdewegh 1984). Different computer programs use different methods. The GAS™ programme incorporates Hodge weighting:

$$W=\frac{4\{2n-3+(1/2)^{n-1}\}}{3n(n-1)}$$

Other programmes, such as Sibpair™ and Mapmaker Sibs™, treat each set of siblings independently.

4.3.2 ANALYSIS OF AFFECTED SIB-PAIR METHODS

Numerous programs are now available for the analysis of affected sib-pair (ASP) studies. Although they are all essentially based on looking at whether ASPs share more than the expected 50% of their alleles at a disease susceptibility locus, they apply many methods. Some look at IBS sharing rather than IBD sharing. The power of the test depends not only on this aspect, but also on the true mode of inheritance, whether single- or multipoint analysis is used, how many parents are typed, and various correction factors that are incorporated (Davis and Weeks 1997). A lot of the programmes use the maximised lod score method, some applying Holman's method of "maximising within the possible triangle" in order to eliminate implausible results. Different programmes deal with greater than two affected siblings in different ways. Some use a weighting function, whilst others allow the user to select whether just one pair is used, one sibling is

chosen and paired with all other affected siblings in turn, or all possible pairs are used.

Analysis can be performed in two main ways: single-point or multi-point analysis. In the former, one marker is analysed at a time. Because of recombination effects, the further away the marker is from the gene, the less likelihood there is of detecting linkage. As a result some degree of misclassification can occur. In multi-point analysis, because several markers are investigated at once, recombination events can be compensated for. Multipoint methods allow exclusion mapping to be performed in addition to maximum-likelihood mapping (Kruglyak and Lander 1995).

GAS™

GAS™ is a single-point method of linkage analysis. It only analyses families in which data are available from both sets of parents. Hodge weighting is applied to families with multiple affected siblings and Holman's maximum sharing triangle is applied in calculating the maximum lod scores. A two-sided chi-squared test is used to detect deviations from the expected 2:1:0 ibd allele sharing of affected sib-pairs. This decreases the power of this method compared to a lot of other methods. A one sided binomial test is used for looking at 1:0 sharing. When simulation studies were performed by Davis and Weeks, GAS™ was found to have a false positive rate of more than 6% (Davis and Weeks 1997).

THE SIBPAIR™ PROGRAM

This program can be used to estimate ibd allele sharing based on population allele frequencies. It uses a likelihood based test that calculates the lod score for nuclear families by assuming that the parents are heterozygous at the disease predisposing allele

and that affected sibs are homozygous (Knapp et al. 1994). It treats affected siblings independently e.g.trios are treated as two sib-pairs, and sib quads as 3 sib-pairs. The average number of shared and unshared alleles is computed for each sibling and a given proband. The average is calculated from the results of all sibs that could be considered probands. If a parental genotype is unavailable, the program computes the likelihood of each possible genotype for the untyped parents, and calculates the number of alleles shared identical by descent in the affected siblings as an average of all the possible parental genotypes. Data from homozygous parents is omitted. A chi-squared test is then performed on the number of sib-pairs sharing 2 alleles ibd with the number sharing 0 alleles ibd (Trust 1996). This method was used successfully by Julier et al. in defining the insulin IGF2 region (Julier et al. 1991).

MAPMAKER-SIBS

This is a multi-point method of maximum-likelihood mapping. Information is used from all available siblings and parents to infer the IBD distribution at each point along the genome. This means that additional power is gained from analysing the same number of families as single-point analysis. The method is particularly useful in cases where families are incomplete. The programme can analyse multiply affected siblings in different ways. Holman's possible triangle method is applied during MLS calculation (Holmans 1993). The program can be used to give maximum-likelihood data in qualitative traits, exclusion maps, quantitative trait mapping and information content mapping (Kruglyak and Lander 1995).

4.4 RESULTS

The genome screen was performed in two stages. In the first instance 92 families from South India were screened for 293 markers, of which 14 failed to amplify. The markers had a mean sex-averaged interval of 11.7cM (range 0.2-40.2). 39 markers had intervals greater than 20cM. The overall length covered was 3415cM. A second screen was performed on all markers from the first round that showed p-values or maximum likelihood estimates that were suggestive of possible linkage, in the hope of replicating any true linkages.

Evidence for linkage was evaluated in two ways – a chi-squared statistic was calculated by comparing the observed and expected allele sharing identical by descent and two-point linkage methods were used to calculate maximum likelihood estimates of the odds ratio. Initially GAS™ was used in the analysis, but due to the discovery that it had been found to be unreliable, we changed to Sib-Pair™ during the second round of the analysis, re-analysing all previous data at that point (Davis and Weeks 1997). Multipoint analysis was also performed using Mapmaker Sib™ for all the first round results.

Ninety-six nuclear families from South India were used in the second round. They were screened for markers where the MLS was greater than 1 or the p value less than 0.10 on Sib-Pair™ (or on GAS™ in cases where Sib-Pair™ analysis had not yet been performed). Ruby Siddiqui and Karen Young screened the families from Vishakapatnam using markers in which the lod scores in both the first and second rounds had been greater than 1. They also screened flanking markers around D10S197 and D2S125.

The results of the first round genome screen on the South Indian families are shown in full in the appendix. Table 4.1 summarises the results of GAS™ and Sib-Pair™

	1st round			2nd round	
Marker	**p-value**	**Lod score**	**MMS score**	**p-value**	**Lod score**
D1S229	0.041	0.653	0.525	0.5	0
D2S165	0.0009	2.103	1.279	0.274	0.079
IL-1α	0.007	1.29	0.937	0.5	0
D2S125	0.028	0.788	1.217	0.155	0.224
D3S1279	0.028	0.793	1.306	0.39	0.017
D4S1565	0.051	0.581	0.633	0.069	0.476
D5S422	0.024	0.845	0.787	0.29	0.77
D5S408	0.03	0.766	0.864	0.5	0
D6S273	0.046	0.618	0.075	0.013	1.064
ESR	0.019	0.939	0.698	Failed amplification	Failed amplification
D7S692	0.025	0.837	1.233	Failed amplification	Failed amplification
CFTR	0.001	1.937	0.059	0.5	0
D7S550	0.01	1.197	0.757	0.3	0.06
D8S286	0.01	1.161	0.654	0.5	0
D8S257	0.046	0.619	0.787	0.357	0.029
D8S556	0.014	1.041	0.535	0.331	0.04
D9S257	0.007	1.319	1.124	0.5	0
D9S176	0.053	0.562	1.094	0.104	0.343

Table 4.1 Results from 1st and 2nd rounds of the genome screen in Tamil Nadu analysed by Sib-Pair™ (chromosomes 1-9)

	1st round			2nd round	
Marker	**p-value**	**Lod score**	**MMS score**	**p-value**	**Lod score**
D10S197*	0.127	0.283	0.073	0.008	1.244
D12S43	0.016	0.992	1.215	0.5	0
D12S95	0.037	0.689	1.468	0.5	0
D12S366	0.037	0.692	0.461	0.354	0.03
D13S153	0.021	0.897	1.447	0.497	0
D15S117	0.048	0.601	0.49	0.06	0.525
D16S515	0.05	0.576	0.414	0.5	0
D16S516	0.007	1.312	1.98	0.5	0
D16S289	0.016	0.992	2.34	0.5	0
D16S422	0.004	1.541	1.357	0.5	0
HOX2B	0.017	0.985	0	0.099	0.361
DXS451	0.05	0.586	0.171	0.389	0.017

*repeated before sib-pair analysis completed based on GAS result of p=0.091 and maximum lod score = 0.4

Table 4.1 (continued) Results from 1st and 2nd rounds of the genome screen in Tamil Nadu analysed by Sib-Pair™ (chromosomes 10-X)

analysis on first rounds markers that were rescreened in the second round, and Fig. 4.3 shows two maps produced by Mapmaker Sibs™ on the results from chromosomes 2 and 10. There was no evidence of a major gene, but the results that stood out as particularly interesting were three consecutive markers on chromosome 16, where the lod score for one of the markers reached 2.34.

No markers stood out in the second screen, although 2 markers, D6S273 and D10S197, had lod scores over 1. Table 4.2 shows the combined results of the first two rounds of the South Indian study. The chromosome 16 results were now completely insignificant apart from Mapmaker Sib™ analysis on D16S289 (lod score 1.419). The markers that now stood out were D2S165 (lod score of 1.47 by Sib Pair™), D6S273 (lod score of 1.75 by Sib Pair™), D10S197 (lod score of 1.25 by Sib Pair™) and HOX2B on chromosome 17 (1.39 by Sib Pair™). None of these scores are very remarkable even after multipoint analysis, but may point to the presence of minor genes.

Table 4.3 shows the combined results of the South Indian screens with the Vishakapatnam study, which have recently become available (Ruby Siddiqui – personal communication). Flanking markers were used for D2S125 and D10S197. Mapmaker Sib analysis™ produced a lod score of 3.006 with D10S197. It is unlikely to represent a major gene effect since it required analysis of 288 families to see much of an effect (over 316 alleles were available for analysis after allowing for non-paternities and homozygous/intercross families). However, it still represents a very exciting result- especially in view of a nearby candidate gene (see below).

4.4.1 OTHER COMMENTS ON THE SCREEN

There were 15 families in which non-parentage was present in the first round. These

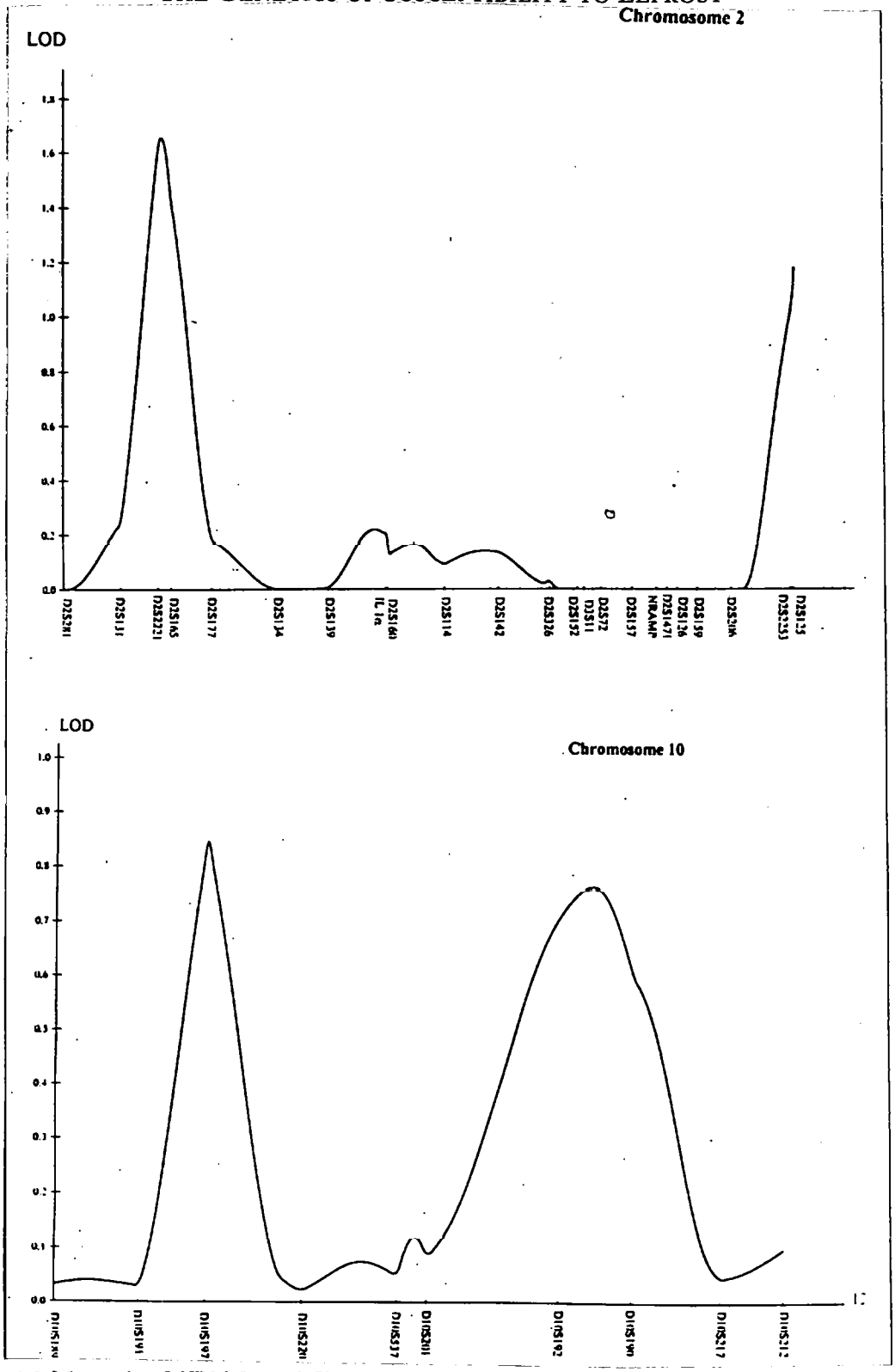

Fig.4.3 Mapmaker Sib™ Maps of Chromosmes 2 and 10 results from the first round screen in Tamil Nadu

Marker	p-value	Lod score
D2S165	0.005	1.47
D2S125	0.016	1.0
D4S1565	0.016	1.0
D6S273	0.002	1.75
D7S692*	0.025	0.84
D7S550	0.023	0.87
D9S176	0.019	0.93
D10S197	0.008	1.25
D15S117	0.022	0.87
HOX2B	0.006	1.39

* Failed amplification in round 2

Table 4.2 Combined Sib Pair™ Results of Rounds 1 and 2 of Genome Screen in Tamil Nadu where the results show one or more score of interest (courtesy of R.Siddiqui)

Marker	p-value	Lod score	MMS
D2S165	0.011	1.14	0.609
D2S125	0.007	1.323	0.929
D4S1565	0.145	0.243	0.284
D6S273	0.079	0.939	0.671
D7S530	0.039	0.671	0.558
D10S1653	0.0004	2.44	1.646
D10S548	0.002	1.927	2.519
D10S197	0.002	1.87	3.006
HOX2B	0.006	1.392	0

Table 4.3 Combined Results of Genome Screen in South India (Tamil Nadu and Vishakapatnam) (courtesy of R.Siddiqui)

families were all replaced. In the second round 14 families were identified with non-parentage. These families were excluded from the final analysis. The overall non-paternity rate in this population was 15.3%, corresponding well with previous data in South India (Fine et al. 1979). The rate was significantly higher in the families from Vishakapatnam at 32% (R. Siddiqui – personal communication). There is a large degree of consanguinuity in South India (see table 3.4). As a result of this, the number of families that were informative was decreased. Intercross parents and cases of homozygosity were frequent, causing, on average, a 25% reduction in information.

4.4.2 POTENTIAL CANDIDATE GENES FOR FURTHER INVESTIGATION

The reason for performing a genome-wide search was to identify regions that may be linked to disease susceptibility genes. We did not find any evidence for major leprosy susceptibilty genes in this screen, although the recent result obtained by Ruby Siddiqui using the families from Vishakapatnam is very exciting. It may be that there is a gene of moderate effect in the region of D10S197. However, the finding of a number of low p-values and lod scores above 1 after combining the first and second screen round results, some of these being further reinforced by the results in Vishakapatnam, may indicate the presence of some minor genes in the vicinity of these markers.

There are two main approaches to identifying genes. These are fine mapping, including chromosome walking and jumping, and the identification of candidate genes followed by case-control studies. Fine mapping is a very expensive and labour intensive procedure, and therefore only worthwhile in cases where there is a strong suggestion of linkage and no candidate genes are available. Only a small percentage of the estimated

65-80,000 genes in the human genome have been identified (Strachan and Read 1996). Despite this, there are frequently a large number of candidate genes in any given region. Best guess policies based on knowledge of the function of the given gene, together with information of currently identified polymorphisms are used to choose whether any of these are worth pursuing. Below I have outlined a few potential candidate genes that may be worth pursuing further based upon the results of our genome screen on leprosy. Table 4.4 summarises these.

CHROMOSOME 2

There are a number of candidate genes in the vicinity of D2S165 that may play a role in influencing susceptibility to infection. The T-lymphocyte activation gene on 2p12 encodes a protein that is expressed in T lymphocytes, the liver and mature neurons (Arai et al. 1992). It is related to the dual function ecotropic retrovirus receptor/cationic amino acid transporter (CAT1). The lymphocyte isoform, CAT2β, is a Na^+ independent basic amino acid transporter with a relatively high affinity for arginine (Kavanaugh et al. 1994). The increased uptake of arginine may be related to its role as a precursor of nitric oxide, an important intermediary in mycobacterial killing.

Some other candidate genes of interest include Catenin (cadherin associated protein), Calmodulin and the Ig kappa light chain gene cluster. Catenin is a peripheral cytoplasmic protein that mediates the connection of cadherin to the actin filament network (Kemler 1993). (See Chromosome 16 candidate genes for further details). It is possible that variations in this connection could affect phagocytic properties of the cell. Calmodulin is involved in the regulation of nitric oxide biosynthesis, so a polymorphism in this gene

Marker	Position	Nearby candidate genes (Name and position)
D2S165	2p12 43.081Mb	T-lymphocyte activation gene, Catenin (CAP-R 97.5+/-13.3), Calmodulin (CALM1), Ig kappa light chain gene cluster
D2S125	2q37 253.365Mb	NRAMP1 (231.3+/-4.2 Mb)
D6S273	6p21-22 37.344Mb	MHC (e.g. HLA-DMB 37.805Mb), TNF (37.805Mb), numerous others
D10S197	10p13 20.312Mb	Macrophage Mannose Receptor (MRC1 25.9 +/-3.5)
D16S516	16q22-24 92.615Mb	Cadherin (CDH13 96.1-97.8)
D16S289	16q22-24 94.067Mb	Cadherin (CDH13 96.1-97.8)
HOX2B	17q21-22 50.200Mb	Nerve Growth Factor Receptor (NGFR 53.9+/-5.3)

Table 4.4 Candidate genes of markers identified from the leprosy genome screen that might be worth pursuing further

(Positions obtained using *http://gdbwww.gdb.org* and *http://cedar.genetics.soton.ac.uk/public-html/*)

could well influence host defence against mycobacteria (Bredt and Snyder 1990).

Variations in the Ig kappa light chain would probably influence humoral immune responses rather than cellular ones, but could still play a crucial role in the control of infections.

Candidate genes in the vicinity of D2S125 include NRAMP1, which is dicussed further in chapter 6.

CHROMOSOME 6

D6S273 is right in the middle of the Major Histocompatibility Complex, close to numerous potential candidate genes including the Tumour Necrosis Factor gene (discussed in chapter 5).

CHROMOSOME 10

The candidate gene that stands out in the vicinity of D10S197 is the human macrophage mannose receptor. This is a C-type lectin involved in the recognition and binding of high mannose structures present on the surface of potentially pathogenic organisms. The receptors are present on macrophages and on the endothelial cells of the liver. They have been found to mediate phagocytosis of mycobacteria following recognition of lipoarabinomannam on the cell surface (Kang and Schlesinger 1998). Interestingly, the receptor is upregulated by IL-4 and IL-13, which might explain the association of multibacillary leprosy with a Th2 response (DeFife 1997). No polymorphisms have been identified to date.

CHROMOSOME 16

Cadherin is a very strong candidate gene in the region of D16S516 and D16S289. The cadherins are a family of cell-cell adhesion molecules involved in calcium dependent intercellular interactions. Classically they comprise N-, R-, M-, P-, and E-cadherin, present in nervous, retinal and nervous, skeletal muscle, placenta and epithelial tissues, respectively. They are glycoproteins with highly conserved cytoplasmic domains, a transmembrane region and an extracellular domain involved in homophilic calcium dependent adhesion. Recently it has been shown that E-cadherin is the receptor for internalin, the surface protein used by Listeria monocytogenes to gain entry into epithelial cells (Mengaud et al. 1996). H- (heart) and T- (truncated) cadherin are present on 16q24, near to D16S516. T-cadherin is present on many tissues including many neuronal cells, frequently in close association with N-cadherin. To date the only polymorhisms known in cadherin genes are in E-cadherin on 16q22.1. They affect codons 115, 133, 582 and 2797. The latter has a relatively high frequency and is situated 54 nucleotides downstream of the TAG stop codon (Becker et al. 1995).

CHROMOSOME 17

HOX2B, a homeobox gene, is located near to the Nerve Growth Factor Receptor. This is a potential candidate gene in view of the neurotropism of *M. leprae*. It is possible that the bacteria enter the Schwann cells using this receptor.

4.5 CONCLUSION

In conclusion, no major susceptibility gene for leprosy has been identified in this population from South India. A study of this size, in which all the genotypes are

informative, has a power of 99.9%, so the chance that we have missed a major gene is very small (Davies et al. 1994). It could be that there is a major gene causing susceptibility to lepromatous leprosy that we have missed since the vast majority of the cases we studied had tuberculoid leprosy. Alternatively, it may be that, despite the findings in segregation analyses, there is no major leprosy susceptibility gene. Instead, several minor genes may be controlling susceptibility, which may be detectable using association studies. In leprosy the λ_s has been calculated as 2.38 (Risch 1987). Some of this effect is already known to be due to the MHC, which does not leave a tremendous amount of room for a major gene. In fact it seems unlikely that the λ_s of any infectious disease is going to be large. Hence, it may be that in future genome screens for infectious diseases may prove more useful as a means of identifying potential candidate genes rather than major genes.

The recent finding of a lod score of 3.006 at D10S197 when the results of 288 families were merged and extra markers mapped suggests the possibility of a gene of moderate effect in this area (R.Siddiqui – personal communication). These results are particularly exciting in view of the presence of the macrophage mannose receptor in the close vicinity of this microsatellite. Case control studies will be performed if a polymorphism is identified in the gene or its promoter region. Transmission disequilibrium studies are also planned.

Richard Bellamy has recently completed a tuberculosis genome screen on sputum positive individuals from the Gambia and South Africa (R. Bellamy – personal communication). Since both tuberculosis and leprosy are caused by mycobacteria that require intracellular growth, one might have expected the two studies to identify identical

regions. Both screens were of similar sizes in terms of the number of sib-pairs and microsatellites studied. Neither identified a major gene, or evidence of strong linkage to the MHC. In the TB study the regions identified with a potential minor effect were on chromosomes Xq and 15p. The former region had already been identified as an area of potential interest in leprosy in view of the raised male to female ratio of disease and an excess of red-green colour-blind individuals amongst leprosy patients (Shwe 1992). We found no evidence for linkage to either of the regions identified in the TB screen.

Some problems associated with genome screens may have affected our results to a degree. Genetic heterogeneity can cause problems in linkage studies. We made sure that all our patients were from one region. Although they were of the same ethnic group (apart from one family who were half Tamil and half Malay), there is evidence that caste group is a strong determinant of genetic profile (Pitchappan et al. 1997). Over 25% of our families were of the Padaychi caste group, but most were from diverse groups, so it is possible that we have missed a gene due to heterogeneity.

Infectious diseases can pose a special problem in this type of study. Not all genotypically susceptible people will get disease. This incomplete penetrance is mainly due to lack of exposure, and the affected sib-pair method circumvents this by only including affected individuals. A large infecting dose in an individual may be sufficient to overcome host defences as may be seen in tuberculosis. Temporary immunosuppression, due to factors such as famine, may allow individuals without the susceptibility gene to be included. This can result in a decrease in the power of the study. Another factor that is difficult to control for is environmental exposure to other mycobacteria, which may

affect both susceptiblity and phenotype.

Because of these problems, and the fact that different genes may be playing a role in different ethnic groups, there are plans to repeat the genome screen in other populations. Sample collection from sibling pairs and their parents is well underway in a population from Malawi. It will be interesting to see whether the findings with D10S197 are repeatable. This population will also be of interest as there are a greater proportion of multibacillary cases than in India. In due course new approaches to gene identification will become available that will make the process of searching for susceptibility genes a great deal more powerful, and may make sample collection more straightforward (Risch and Merikangas 1996).

Above I have discussed some candidate genes that might be worth investigating further based on the results of the genome screen. The association of late onset Alzheimer's disease with ApoE4 provides an example of how evidence for linkage on family studies may be very weak, but allelic association may be very marked (Liu et al. 1996). In the following two chapters I will discuss a few candidate genes that I looked at prior to the completion of the genome screen. These were chosen on the basis of knowledge of their function.

CHAPTER 5

5 CANDIDATE GENE STUDIES PART 1:CYTOKINES

5.1 INTRODUCTION

Cytokines are soluble molecules that can be thought of as the hormones of the immune system, the pattern of cytokine production determining the subsequent immune response. Their production is aimed at protecting the host from toxic inflammatory agents and microbial invasion, but at times there is overproduction of one type of cytokine which may tip the balance in such a way that immunopathology is produced. Cytokines are secreted by lymphocytes, monocytes and macrophages. They have pleiotropic effects and act in both a paracrine and an autocrine fashion. Many of the cytokine genes have now been identified and their actions clarified. Genetic differences in the promoter sequences of cytokine genes may alter the binding of regulatory factors and hence affect the level of transcription of cytokines (Jain et al. 1995). An example of this is clearly demonstrated by work on polymorphisms of tumour necrosis factor in malaria (McGuire et al. 1994).

Leprosy has a diverse clinical and immunological spectrum with a number of complications that may be attributable to variations in cytokine production (see 1.5.1). The Th1/Th2 response plays a major role in determining the outcome of infection with *M. leprae*. Although a Th1 response appears to protect the individual from widespread bacterial dissemination, the same response can result in tissue damage causing both skin and nerve pathology. A Th2 type response allows bacterial growth to occur leading to

lepromatous leprosy. Th1 cells downregulate Th2 cells and vice versa, which tends to polarise the final response (Powrie 1993). In tuberculoid patients, interferon-γ, interleukin-2, TNF and interleukin-12 predominate, whilst interleukins-4, 5, 6, 10 and 13 are seen in lepromatous leprosy patients (Ottenhoff 1994), (Yamamura et al. 1992) and (Misra et al. 1995). In Type 1 leprosy reactions there tends to be a predominantly Th1 response. Despite the fact that Type 2 leprosy reactions only occur in people with lepromatous or borderline lepromatous disease, it appears that during the acute phase of the reaction, Th1 responses predominate (Rao and Rao 1987).

I have looked at three polymorphic cytokines to see if they are involved in determining the clinical manifestations of the disease. The cytokines studied were interleukin-4 (IL-4), interleukin-10 (IL-10) and tumour necrosis factor (TNF). In the remainder of this chapter I will discuss the characteristics of these cytokines, their association with various diseases, and the results of case control studies performed on them using samples from patients from India and Mali. Table 5.1 briefly summarises the candidate genes described in this chapter and Chapter 6, and Table 5.2 summarises the samples used in each study.

5.2 INTERLEUKIN-4

5.2.1 BACKGROUND

Interleukin-4 is an 18-20 kD glycoprotein produced by activated T lymphocytes, basophils and mast cells (Seder et al. 1992). The gene is located in the cytokine cluster on the long arm of chromosome 5. Its main immunoregulatory function is in induction of

Gene	Known associations with infectious diseases	Some immunological effects
IL-4	? Schistosmiasis (intensity of infection linked to 5q31-33)	Promotes TH2 response Inhibits macrophage activity
IL-10	Hepatitis B Cervicits Cervical intraepithelial neoplasia 1	Promotes TH2 response Decreased reactive intermediates
TNF	Cerebral malaria, Leishmaniasis, Leprosy, Fatal meningococcal disease	Promotes granuloma formation Activates macrophage killing
VDR	TB, Hepatitis B, Leprosy	Promotes TH2 response
NRAMP1	Salmonella, Leishmania & mycobacterial infections in mice. TB in humans	Early intramacrophage killing

Table 5.1Candidate Genes discussed in this thesis and some of the evidence to suggest that these candidate genes are worth studying in leprosy

Gene	Polymorphisms studied	Population studied
IL-4	-590C/T	Tamils and Bengalis
IL-10	-1082G/A -592C/A microsatellite	Mali
TNF	-238G/A -308G/A	Tamils
VDR	Taq 1 site	Tamils and Mali
NRAMP1	469+14G/C 1729+14del4 microsatellite	Tamils and Mali

Table 5.2 Summary of Candidate genes studied in this thesis, with the population studied

(IL = interleukin, TNF = tumour necrosis factor, VDR = vitamin D receptor, NRAMP1 = human natural resistance associated macrophage protein 1)

immunoglobulin isotype switching to IgE expression and induction of Th2 cells, which regulate humoral immunity, eosinophilia and inflammatory macrophage deactivation (Song et al. 1996). It also has anti-tumour activity in animal models. Giving IL-4 at the time of an infection causes a Th2 type response (Chatelain et al. 1992). Anti IL-4 antibodies result in inhibition of IL-10 production, suggesting that IL-4 is necessary before IL-10 can be produced. Interleukin-4 is a B cell activating factor resulting in greatly increased expression of class II MHC on B cells, priming them to respond to anti IgM and to express the FcεRII receptor, CD23 (Austyn and Wood 1993). By downregulation of IL-2, it blocks proliferation and interferon gamma production by mononuclear cells. A high affinity IL-4 receptor is present on nearly all types of cell. IL-4 profoundly inhibits the activity of the TNFα promoter and protein production, dampening down the immune response (Rhoades et al. 1995). Macrophage function is markedly reduced, including the production of reactive oxygen intermediates.

5.2.2 PROMOTER POLYMORPHISMS OF THE IL-4 GENE

Rosenwasser found a C to T transition within the IL-4 promoter region at –590 base pairs from the open reading frame. This was associated with a significant increase in the serum IgE levels in 44 Jewish asthmatic and atopic individuals from Colorado (Rosenwasser et al. 1995). These findings were not replicated in Australian or British subjects apart from a weak association of the polymorphism with specific IgE to housedust mite in the Australians (Walley and Cookson 1996). Song et al. identified 8 other IL-4 polymorphisms between positions -306 and +55. Although some of these may not be present at polymorphic frequencies, at least two appear to be functional. An A to G transition at –81 in an OAP_{40} site markedly enhanced trancriptional levels. It is

thought that the transition makes the OAP_{40} element a higher affinity binding site for certain proteins. In humans the polymorphism may lead to overexpression of IL-4 which then biases cells towards the Th2 end of the spectrum (Song et al. 1996).

5.2.3 IL-4 IN DISEASE

BALB/c mice are intrinsically susceptible to infection, whereas B10.D2 mice are intrinsically resistant to infection with Leishmania major. *In vitro* experiments have shown that BALB/c T cells acquire a Th2 type phenotype whilst B10.D2 cells acquire a Th1 type response, and that the former is due to greater initial production of IL-4 (Guler et al. 1996). Both IL-4 and IL-10 inhibit the IFNγ mediated microbicidal effect of macrophages against amastigotes of leishmaniasis in BALB/c mice. Administration of IL-4 antibodies to susceptible mice within the first week of infection cured them of this otherwise lethal infection, whereas recombinant IL-4 given to resistant mice made them susceptible to leishmanisis, schistosomiasis, listeria and trichinella (Sadick et al. 1990). The usual IgE response to Nippostrongylus brasiliensis is ablated when anti IL-4 antibodies are administered to mice prior to challenge with the parasite (Finkelman et al. 1990).

It has long been recognised that atopic conditions tend to run in families and there is good evidence for heritability of IgE levels (Gerrard et al. 1978). Since IL-4 is the cytokine that mediates the switch from IgM to IgE production, it is a good candidate gene for both atopic and parasitic conditions. There have been reports that the IL-4 gene is overexpressed in cells isolated from atopic individuals (Song et al. 1996). Quantitative trait analysis of asthmatic Amish families has shown that the IL-4 gene region on 5q31-

q33 is tightly linked to serum IgE levels and susceptibility to asthma (Marsh et al. 1994). These findings would be consistent with the fact that the production of IgE by T cell clones is directly related to their ability to produce IL-4 (Splawski et al. 1993) and that cells from atopic individuals have high levels of expression of IL-4 (Kay et al. 1991). The same genetic region has been linked to IgE levels and bronchial hyperresponsiveness (Meyers et al. 1994) and (Postma et al. 1995). A genome screen on some Brazilian families demonstrated that this region is also important in determining the intensity of infection with Schistosoma mansonii (Marquet et al. 1996). Despite the above studies, neither a genome wide search for asthma susceptibility genes in Australians nor a study on asthmatic families from Finland found evidence of linkage between chromosome 5q markers and serum IgE levels or asthma (Daniels et al. 1996) and (Laitinen et al. 1997).

As mentioned above, lepromatous leprosy patients have been found to lie at the Th2 end of the immunological spectrum, with high levels of IL-4 in the tissues. IgE specific for Bcg antigen was significantly elevated in patients with tuberculosis and leprosy compared to controls from East Java. The elevation of IgE in both diseases may be due to a shared antigen stimulating the response (Yong et al. 1989).

5.2.4 IL-4 RESULTS

The –590 IL-4 polymorphism was typed by oligonucleotide specific hybridisation in cases and controls collected by Dr. S. Roy from Calcutta, and also in a selection of cases and controls from the samples collected by myself in South India. Results are shown in Tables 5.3 and 5.4. No significant association was found between the different polymorphisms and either susceptibility to leprosy per se, or to leprosy type. Nor was

Genotype	Total Controls	MB cases	ENL Cases	LL Controls
11	59 (75.6%)	79 (79.0%)	38 (74.5%)	26 (86.7%)
12	18 (23%)	16 (16.0%)	10 (19.6%)	3 (10.0%)
22	1 (1.3%)	5 (5.0%)	3 (5.9%)	1 (3.3%)
Total	78	100	51	30

Table 5.3 Interleukin-4 –590 polymorphism and leprosy in South India

(MB = multibacillary cases, ENL = erythema nodosum leprosum cases. LL Controls = lepromatous leprosy controls who had not developed ENL after at least 1 year of therapy. 1 = wild type IL-4 -590 allele, 2 = mutant IL-4 -590 allele).

3x2 χ^2 (2d.f.) =3.01 p=0.222 for total MB cases versus total controls

3x2 χ^2 (2d.f.) =1.69 p=0.430 for ENL cases versus Lepromatous leprosy controls

Controls are in Hardy-Weinberg equilibrium

Genotype	Controls	Tuberculoid	Lepromatous	Total Cases
11	101 (60.8%)	63 (58.9%)	66 (54.5%)	129 (56.6%)
12	51 (30.7%)	37 (34.6%)	45 (37.2%)	82 (36.0%)
22	14 (8.4%)	7 (6.5%)	10 (8.3%)	17 (7.5%)
Total	166	107	121	228

Table 5.4 Interleukin-4 –590 polymorphism and leprosy in Bengal

(1 = wild type IL-4-590 allele, 2 = mutant IL-4-590 allele)

3x2 χ^2 (2d.f.) =0.52 p=0.770 for tuberculoid cases versus lepromatous leprosy cases

3x2 χ^2 (2d.f.) =1.20 p= 0.549 for total cases versus controls

Controls are in Hardy-Weinberg equilibrium

there an association in the South Indians with susceptibility to erythema nodosum leprosum. The mutant allele was less frequent in the South Indian population than in the Bengalis.

5.2.5 DISCUSSION

By enhancing Th2 responses, the individual's resistance to extracellular infections such as encapsulated bacteria and helminth infections is increased. A pay-off for this is that the individual becomes more susceptible to infections with intracellular organisms. Because of its role in the Th1/Th2 response, IL-4 may play an important role in determining susceptibility to infectious diseases, and it may even be that the high prevalence of atopy and asthma is a direct result of selective pressures driven by infectious diseases. It is possible that polymorphisms of IL-4 may promote greater fecundity by enhancing the Th2 response in pregnancy (Wegmann et al. 1993), which may also have shaped the distribution of various alleles and hence the observed disease patterns in different populations.

There is a strong association between Th1 and Th2 responses and the two extreme clinical forms of leprosy. Based upon the role that IL-4 appears to play in shaping this response, one might have expected an excess of mutant alleles in the lepromatous compared with the tuberculoid cases. A non-significant trend in this direction was seen amongst the Bengali population with the –590 IL-4 polymorphism. Th2 responses predominate in lepromatous leprosy, but during acute erythema nodosum leprosum a transient increase in T cell activity occurs consistent with a Th1-type response. A comparison between lepromatous cases who went on to get ENL with those that had

not done so after at least a year of treatment, failed to show a significant difference in IL-4 –590 genotypes between the two groups. If anything there was a trend towards an excess of mutant alleles amongst the ENL cases, the reverse of what might have been expected. Our study was performed in two distinct populations, the difference being clearly apparent in terms of allele frequencies between the two groups, but both failed to show any association with the polymorphism studied.

We only typed a single IL-4 polymorphism. It may be that the polymorphism that we looked at is not responsible for variations in IL-4 expression after all. This may be because there is another unlinked polymorphism in the same region and it would be of interest to investigate these variants further. Interestingly, Blackwell et al. found that the Th2 cluster on chromosome 5 shows linkage to the ability to mount an immune response to mycobacterial antigens in Brazilian families. They found no evidence for linkage of two polymorphisms of IL-4 to leprosy per se (Blackwell et al. 1997).

Other genes may be responsible for the varied expression of IL-4. With the numerous cytokines involved in determining the immune response, there may be complex interactions occurring at both genetic and environmental levels that determine where a patient falls on the clinical spectrum. An IL9 polymorphism was found to be associated with serum IgE in an English study (Doull et al. 1996), which could explain the findings of Marsh (Marsh et al. 1994). Another area of interest that might be worth investigating is the IL-4 receptor gene (IL-4Rα) on chromosome 16. Although studies have ruled out linkage of this region with Job's syndrome of hyper IgE production (Grimbacher et al. 1998), a human variant (Ile50) has been associated with susceptibility to atopic asthma and raised total serum IgE levels in a Japanese population. In animal

studies the Ile50 variant was found to significantly upregulate receptor response to IL-4, and hence IgE production (Mitsyasu et al. 1998).

5.3 INTERLEUKIN-10

5.3.1 BACKGROUND

IL-10 is a 36kD covalent homodimeric protein that is secreted by T cells, B cells, and activated macrophages and is coded for on chromosome 1 (Eskdale et al. 1997). It may be expressed by some CD4 and CD8 Tcell clones and by monocytes, macrophages, activated B cells and keratinocytes. The production of Th1 type cytokines from T cells, macrophages and monocytes is reduced in the presence of IL-10, favouring a Th2-like response that allows the survival of pathogens (Sher et al. 1991) and (Fiorentino et al. 1991). IL-10 also downregulates class II MHC expression by monocytes and macrophages, thus inhibiting antigen presentation and resulting in suppression of chronic granulomatous inflammatory responses (Herfarth et al. 1996). However, class II MHC expression on B cells is upregulated, causing them to proliferate and differentiate into plasma cells, hence increasing the production of IgM, IgG and IgA levels. Mast cell proliferation and differentiation is promoted by IL-10. By blocking IFNγ activation and TNFα production IL-10 inhibits the synthesis of nitric oxide, resulting in decreased resistance to intracellular parasites (Sher et al. 1991).

In the presence of IL-2, 4 or 7 it acts as a costimulator of proliferation of thymocytes and T cells. Unlike IL-4 there is no class switching of lipopolysaccharide stimulated B cells. It has been implicated in autoimmune disease, malignancy, and transplant tolerance. Levels of IL-10 appear to be critical in determining the type of immune response that

develops in response to parasitic infections (Sher et al. 1991) and (Mosmann and Moore 1991). The overall effect of IL-10 is decreased Th1-type T cell activity, decreased cytotoxic T lymphocyte mediated lysis, and suppression of macrophage activity.

5.3.2 IL-10 IN DISEASE

IL-10 production is markedly increased in animals with a Th2 type response as occurs in nematode infections e.g. L.major, S.mansonii, T.cruzii, and N.braziliensis. IL-10 deficient mice develop an enterocolitis, which may be due to failure to control normal immune responses against enteric antigens. This would result in chronic inflammation secondary to continual overproduction of Th1 type cytokines, enhanced class II expression, and increased exposure of lymphoid cells to luminal antigens and bacterial cell wall components (Kuhn et al. 1993).

Patients with cervicitis, CIN1, and adenocarcinoma of the cervix have been found to have a higher incidence of the -1082 G allele (high producers of IL-10) and also a higher frequency of the TNFα -308 G allele (low producers of TNFα) compared to control patients ($p=0.04$). This results in a Th2 type response (Majeed et al. 1997). Infection with M.avium induces IL-10 production and it is possible that IL-10 enhances host susceptibility to mycobacteria (Bermudez and Champsi 1993). Several of the IL-10 polymorphisms mentioned below have been associated with protection from hepatitis B carriage following acute infection (Zhang et al personal communication).

5.3.3 IL-10 POLYMORPHISMS

Turner et al. have confirmed the presence of 3 single base pair substitutions in the IL-10 promoter region and investigated the correlation of the polymorphisms with

protein production *In vitro*. The mutations are a G to A substitution at –1082, a C to T substitution at –892, and a C to A substitution at –592. Healthy kidney donors without the –1082 A allele had significantly higher levels of IL-10 than those with the A allele (Turner et al. 1996). The polymorphism lies within an ETS-like recognition site, a transcription factor that has been shown to downregulate IL-2 production and may have the same effect on the binding of IL-10 (Kube et al. 1995) (Romano-Spica et al. 1995). The IL-10 microsatellite has been found to be in a region that decreases promoter function (Kube et al. 1995). Eskdale and Gallagher have speculated that this length polymorphism affects expression of IL-10 (Eskdale and Gallagher 1995).

5.3.4 IL-10 RESULTS

IL-10 typing was performed on the samples collected in Mali using oligospecific typing for the –1082 and –592 polymorphisms, and fluorescently labelled microsatellite typing for the microsatellite. The –892 polymorphism was not studied because previous findings have shown complete linkage disequilibrium between this and the –592 polymorphism in the Gambia. The results are shown in tables 5.5 to 5.13. No significant association was found between the different polymorphisms and either susceptibility to leprosy per se, or to leprosy type or form in this population. Mantel Haenszel tests performed on the results for the four largest ethnic and the four largest regional groups failed to show any association with either the –1082 or the –592 genotypes (GG vs. GA + AA and CC vs. CA + AA respectively).

Because of the potential role of IL-10 in determining differences in Th1 and Th2 responses, comparisons of the genotypes were made between those experiencing no

IL-10 –1082 Type	MB	PB	Controls	Total
GG	85(47.2%)	44(48.4%)	82(44.8%)	211
GA	73(40.6%)	42(46.2%)	78(42.6%)	193
AA	22(12.2%)	5(5.5%)	23(12.6%)	50
Total	180	91	183	454

Table 5.5 Interleukin-10 -1082 polymorphism types in multibacillary (MB) and paucibacillary(PB) cases and controls from Mali (G=wild allele, A= mutant allele)

3x2 χ^2 (2d.f.) =0.86 p=0.651 for controls versus leprosy cases

3x2 χ^2 (2d.f.) =3.21 p=0.201 for PB vs. MB leprosy cases

Comparison of controls versus leprosy cases in the 4 largest regional groups (GG vs GA + AA) 2x2 Mantel-Haenszel test χ^2 =0.04, p=0.849, odds ratio = 1.07 (C.I. 0.68-1.69)

Comparison of controls versus leprosy cases in the 4 largest ethnic groups (GG vs GA + AA) 2x2 Mantel-Haenszel test χ^2 =0.00, p=0.980, odds ratio = 1.03 (C.I. 0.66-1.60)

Comparison of MB versus PB cases in the 4 largest regional groups (GG vs GA + AA) 2x2 Mantel-Haenszel test χ^2 =0.94, p=0.95, odds ratio = 0.89 (C.I. 0.51-1.75)

Comparison of MB versus PB cases in the 4 largest ethnic groups (GG vs GA + AA) 2x2 Mantel-Haenszel test χ^2 =0.06, p=0.799, odds ratio = 0.90 (C.I. 0.5 -1.60)

Controls are in Hardy-Weinberg equilibrium.

IL-10 -1082 Type	LL	TT
GG	61(45.2%)	28(48.3%)
GA	55(40.7%)	25(43.1%)
AA	19(14.1%)	5(8.6%)
Total	135	58

Table 5.6 Interleukin-10 -1082 polymorphism types in cases of lepromatous (LL) and tuberculoid (TT) leprosy cases from Mali (G=wild allele, A= mutant allele)

3x2 χ^2 (2d.f.) =1.11 p=0.574 for LL vs. TT cases

Comparison of LL versus TT cases in the 4 largest regional groups (GG vs GA + AA)

2x2 Mantel-Haenszel test χ^2 =0.07, p=0.794, odds ratio = 1.22 (C.I. 0.49 – 3.03)

Comparison of LL versus TT cases in the 4 largest ethnic groups (GG vs GA + AA) 2x2

Mantel-Haenszel test χ^2 =0.00, p=0.946, odds ratio = 0.91 (C.I. 0.4 - 2.07)

Controls are in Hardy-Weinberg equilibrium.

IL-10 -1082 Type	No reaction	Type 1 reaction	Type 2 reaction
GG	59(47.2%)	21(51.2%)	49(46.7%)
GA	57(45.6%)	15(36.6%)	43(41%)
AA	9(7.2%)	5(12.2%)	13(12.4%)
Total	125	41	105

Table 5.7 Interleukin-10 polymorphism types in cases of leprosy reaction from Mali (G=wild allele, A= mutant allele)

3x2 χ^2 (2d.f.) =1.6 (2d.f.) p=0.450 for no reaction vs. Type 1 reaction

3x2 χ^2 (2d.f.) =1.89 (2d.f.) p=0.389 for no reaction vs. Type 2 reaction

3x2 χ^2 (2d.f.) =2.33 (2d.f.) p=0.311 for Type 1 or Type 2 reaction vs. noreaction

Controls are in Hardy-Weinberg equilibrium

IL-10 -592 Type	MB	PB	Controls	Total
CC	55(30.7%)	26(28.6%)	66(33.7%)	147
CA	79(44.1%)	43(47.3%)	85(43.4%)	207
AA	45(25.1%)	22(24.2%)	45(23%)	112
Total	179	91	196	466

Table 5.8 Interleukin-10 -592 polymorhism types in multibacillary (MB) and paucibacillary(PB) cases and controls from Mali (allele types are A and C)

3x2 χ^2 (2d.f.) =0.73, p=0.693 comparing total cases (MB+PB) vs. controls

3x2 χ^2 (2d.f.) =0.25, p=0.884 comparing MB vs.PB cases

Comparison of total cases (MB+PB) vs. controls in the 4 largest regional groups (CC vs. CA + AA) 2x2 Mantel-Haenszel test χ^2 =0.07, p=0.795, odds ratio = 0.92 (C.I. 0.56 - 1.49)

Comparison of total cases (MB+PB) vs. controls in the 4 largest ethnic groups (CC vs. CA + AA) 2x2 Mantel-Haenszel test χ^2 =0.14, p=0.704, odds ratio = 0.90 (C.I. 0.57 - 1.42)

Comparison of MB versus PB cases in the 4 largest regional groups (CC vs. CA + AA) 2x2 Mantel-Haenszel test χ^2 =0.34, p=0.559, odds ratio = 1.27 (C.I. 0.64 –2.58)

Comparison of MB versus PB cases in the 4 largest ethnic groups (CC vs. CA + AA) 2x2 Mantel-Haenszel test χ^2 =0.63, p=0.428, odds ratio = 1.34 (C.I. 0.70 - 2.57)

Controls are in Hardy-Weinberg equilibrium.

IL-10 -592 Type	LL	TT
CC	44(32.8%)	17(29.3%)
CA	58(43.3%)	25(43.1%)
AA	32(23.9%)	16(27.6%)
Total	134	58

Table 5.9 Interleukin-10 -592 polymorphism types in cases of lepromatous (LL) and tuberculoid (TT) leprosy from Mali (allele types are A and C)

3x2 χ^2 (2d.f.) = 0.38, p=0.827 comparing LL vs. TT cases

Comparison of LL vs. TT cases in the 4 largest regional groups (CC vs. CA + AA)

Mantel-Haenszel test 2x2 χ^2 =1.49, p=0.223, odds ratio = 2.06 (C.I. 0.71 – 6.42)

Comparison of LL vs. TT cases in the 4 largest ethnic groups (CC vs. CA + AA) Mantel-Haenszel test 2x2 χ^2 =0.5, p=0.479, odds ratio = 1.48 (C.I. 0.60 - 3.73)

IL-10 -592 Type	No reaction	Type 1 reaction	Type 2 reaction
CC	37(29.6%)	13(32.5%)	31(29.5%)
CA	58(46.4%)	13(32.5%)	51(48.6%)
AA	30(24%)	14(35%)	23(21.9%)
Total	125	40	105

Table 5.10 Interleukin-10 -592 polymorphism types in cases of leprosy reactions from Mali (allele types are A and C)

3x2 χ^2(2d.f.) =2.82 p=0.244 for no reaction vs. Type 1 reaction

3x2 χ^2 (2d.f.)=0.17 p=0.921 for no reaction vs. Type 2 reaction

3x2 χ^2 (2d.f.)=0.15 p=0.27 for Type 1 reaction plus Type 2 reaction vs. no reaction

Microsatellite Allele	MB	PB	Controls	Total
124	0	1 (0.6%)	1 (0.3%)	2
130	0	0	1 (0.3%)	1
132	23 (6.8%)	15 (8.9%)	23 (6%)	61
134	20 (6%)	11 (6.5%)	15 (3.9%)	46
136	113 (33.6%)	51 (30.4%)	116 (30.4%)	280
138	50 (14.9%)	20 (11.9%)	61 (16%)	131
140	71 (21.1%)	35 (20.8%)	93 (24.3%)	199
142	31 (9.2%)	20 (11.9%)	35 (9.2%)	86
146	22 (6.5%)	9 (5.4%)	27 (7.1%)	58
148	6 (1.8%)	6 (3.6%)	10 (2.6%)	22
Total	336	168	382	886

Table 5.11 Interleukin-10 microsatellite allele frequencies in multibacillary (MB) and paucibacillary (PB) cases and controls from Mali

There are no significant differences in the allele frequencies between the different categories.

Microsatellite Allele	LL(allele frequency)	TT(allele frequency)
124	0	1 (1.3%)
130	0	0
132	15 (6.4%)	3 (3.8%)
134	12 (5.1%)	4 (5.0%)
136	81 (34.3%)	27 (33.8%)
138	31 (13.1%)	12 (15.0%)
140	49 (20.8%)	15 (18.8%)
142	25 (10.6%)	13 (16.3%)
146	18 (7.6%)	5 (6.3%)
148	5 (2.1%)	1 (1.3%)
Total	236	80

Table 5.12 Interleukin-10 microsatellite allele frequencies in cases of lepromatous (LL) and Tuberculoid (TT) leprosy in Mali

There are no significant differences in the allele frequencies between the different categories.

Microsatellite Allele	No reaction	Type 1 reaction	Type 2 reaction
124	1 (0.4%)	0	0
130	0	0	0
132	19 (8.2%)	6 (7.9%)	13 (6.6%)
134	14 (6.0%)	7 (9.2%)	10 (5.1%)
136	73 (31.5%)	20 (26.3%)	71 (35.9%)
138	32 (13.8%)	11 (14.5%)	28 (14.1%)
140	45 (19.4%)	17 (22.4%)	44 (22.2%)
142	27 (11.6%)	7 (9.2%)	17 (8.6%)
146	14 (6.0%)	6 (7.9%)	12 (6.1%)
148	7 (3.0%)	2 (2.6%)	3 (1.5%)
Total	232	76	198

Table 5.13 Interleukin-10 microsatellite allele frequencies in type 1 and type 2 leprosy reactions from Mali

There are no significant differences in the allele frequencies between the different categories

reaction and those experiencing a type 1, type 2 and types 1 and 2 combined reaction. No significant differences were found between these groups although there was a trend towards those people experiencing both type 1 and type 2 reactions to have a greater number of mutant homozygotes (AA) in the IL-10 – 1082 Group. The IL-10 microsatellite alleles showed no evidence of any particular allele predisposing individuals to, or protecting them from the different forms or types of leprosy, reactions or leprosy per se.

5.3.5 DISCUSSION

As with IL-4, IL-10 probably plays a major role in the Th1/ Th2 response and hence in determining the individual's response to intracellular and extracellular organisms. Despite evidence for functional polymorphisms of IL-10, and involvement in a number of diseases, we have not found any associations between leprosy and these polymorphisms. The trend towards an increase in the IL-10 –1082 AA homozygotes in people experiencing both type 1 and type 2 reactions is interesting. It is consistent with the findings of Turner et al. (see section 5.3.3), given that in those experiencing type 1 reactions, and transiently in ENL patients, there is a marked Th1 type response (Turner et al. 1996) and (Rao and Rao 1987).

It would be interesting to repeat these studies in the samples collected in South India to see if the trend persists or becomes significant. It may be that there are other unlinked functional polymorphisms of IL-10 that have not yet been identified that play a role in determining susceptibility to leprosy or leprosy type. Alternatively, other cytokines may

exert a stronger influence in determining the immune response, and hence determining leprosy disease expression.

5.4 TUMOUR NECROSIS FACTOR

5.4.1 BACKGROUND

The tumour necrosis factors (TNF) are multifactorial cytokines that possess numerous biological activities involving the acute phase response. It has been suggested that TNF may act as an important antigen independent host defence mechanism against infectious disease. Production of TNFα is mainly stimulated by infection and tissue injury, TNFβ by tissue injury.

TNFα (also known as cachectin) is a 157 amino acid polypeptide with a molecular weight of 17kD. It is a trimeric molecule with an antiparallel β-pleated sheet ordered in a "β barrel jelly roll" (Jones et al. 1989). The gene is appprox. 3.6 kb, contains 3 introns and is coded for by genes in the class III region of MHC on the short arm of chromosome 6 between p21.1 and p21.3, approximately 250kb centromeric to HLA-B and 1Mb telomeric to HLA-DR (Dunham et al. 1987). TNFα is produced by activated macrophages in response to a wide range of stimuli such as bacteria and their products, viruses, and other cytokines. It is also secreted by activated T cells, natural killer cells (NK cells), B cells, smooth muscle cells and keratinocytes. There are TNF receptors on all cells, hence TNF has numerous and varied biological effects.

TNFα stimulates leucocytes to adhere to other cells to a greater degree, and to

release cytokines. The expression of class II and IL-2 receptors by T cells is upregulated. TNF, in synergy with IFNγ triggers the activation of both the superoxide and nitric oxide dependent cytotoxic pathways. Macrophages have greater phagocytic activity, whilst NK and CTLs are more effective in their cytotoxic role. TNF in concert with IL-2 results in proliferation of activated B cells and increased antibody expression. TNFα results in increased adhesion of leucocytes to endothelial cells, release of IL-1, 6, 8 and GM-CSF from endothelial cells and increased permeability by loss of fibronectin from the basement membrane. Complement receptor expression is upregulated. All these actions result in increased inflammation and enhanced host antimicrobial activity (Tracey and Cerami 1993) and (Austyn and Wood 1993).

As well as tumoricidal activity and useful effects in viral, bacterial and parasitic infections, TNF plays a role in various forms of pathology. These include induction of tumorigenesis and metastasis, septic shock, cerebral malaria, the Jarisch-Herxheimer reaction and autoimmunity (Beutler and Cerami 1989) and (Vassalli 1992). TNF may contribute to anaemia by causing dyserythropoesis and erythrophagocytosis (Clark and Chaudhri 1988). As with many cytokines, there seems to be a fine line between the beneficial effects of TNF in infections, and pathological effects.

TNF β, also known as lymphotoxin, is a glycoprotein that is produced by activated T lymphocytes usually as a result of MHC restricted antigen presentation. It too is coded by genes in the class III region of MHC in close proximity to TNFα, and is probably derived from a common ancestor. There is 28% homology at amino acid level, and 46% at nucleotide level to TNFα (Turetskaya et al. 1992). TNF β receptors are present on

almost every cell and it has been implicated in a wide range of syndromes and diseases. It is involved in the killing of bystander cells in delayed type hypersensitivity reactions, the induction of necrosis in tumour cells, increased expression of MHC class I and adhesion molecules on endothelial cells, increased activity of polymorphonucleocytes, and resorption by bone osteoclasts. It probably plays an important role in defense against viral, bacterial and parasitic infections (Austyn and Wood 1993).

5.4.2 POLYMORPHISM IN THE TNFα PROMOTER

The biosynthesis of TNF is controlled at many levels. Beutler et al. found that stimulation of monocytes and macrophages with LPS resulted in a 3 fold increase in TNF transcription levels, 100 fold increase in mRNA content and 10,000 fold increase in TNFα protein secretion (Beutler and Cerami 1989). There are numerous regulatory sequences in the 5' region of the gene. These include NFκB elements, GC and TATA boxes that confer a high degree of inducibility on TNF and are all areas where critical nucleotide changes could have significant effects upon transcription (Tracey and Cerami 1993) and (Emery et al. 1993).

Several polymorphisms have been identified in the TNFα promoter region. A G to A substitution within the consensus sequence for the transcription factor AP-2 at position -308 has been associated with higher constitutive and inducible levels of TNF (Wilson et al. 1994). It may function by inhibiting a transcriptional repressor. Homozygotes for this mutation have been found to be at increased risk of cerebral malaria in the Gambia (McGuire et al. 1994). There is an increased frequency of the mutant allele amongst lepromatous compared to tuberculoid leprosy patients in Bengal,

and mucocutaneous leishmaniasis patients have a higher frequency of this allele than controls (Roy et al. 1997) and (Cabrera et al. 1995). Death from meningococcal disease has also been associated with this allele (Nadel et al. 1996).

Another polymorphism has been identified at –238 in the Y box regulatory element of the promoter region. This is a G to A substitution which may alter the curvature of a specific nuclear factor binding site (D'Alfonso and Richiardi 1994). Despite this, there is no evidence that it results in reduced transcription (Pociot et al. 1995). This variant has been associated with an increased risk of severe malarial anaemia in Kenya and the Gambia (D'Alfonso and Richiardi 1994) and (Kwiatkowski, personal communication). Heterozygosity for the -238 mutation has been weakly associated with protection from HIV infection in Uganda (S.Ali, personal communication).

5.4.3 TNF IN MYCOBACTERIAL DISEASE

TNF plays an important antigen independent role in host defense against infectious diseases. This may be mediated by stimulation of effector mechanisms that kill mycobacteria and promote granuloma formation. However high levels can lead to immunopathology including direct damage to myelin and to oligodendrocytes (Selmaj and Raine 1988). TNF plays an important role in mycobacteriocidal and -static mechanisms in macrophages, resulting in the killing of M.avium and restricted growth of *M. tuberculosis* (Bermudez 1988) and (Denis et al. 1990). In mice TNFα enhances the bacteriostatic potential by triggering NO production in IFNγ activated macrophages. Mice that are given anti-TNFα and are infected with *M. tuberculosis* rapidly succumb to disseminated disease (Flynn et al. 1995).

TNF is involved in the formation of granulomas, which control the spread of mycobacterial infections (Vassalli 1992). This appears to be a local phenomenon and has been demonstrated in TB pleuritis (Barnes et al. 1990). Monocytes and macrophages from active TB patients produce higher levels of TNFa than healthy controls and chronic TB patients (Kim et al. 1991). On a more general level, TNF contributes to the cachexia seen in tuberculosis and also to the fever, weight loss and night sweats (Vassalli 1992) and (Tramontana et al. 1995).

5.4.4 TNF AND LEPROSY

TNF probably mediates host defense against mycobacterial diseases by the promotion of granuloma formation and the stimulation of effector mechanisms that kill mycobacteria. Unfortunately these mechanisms may also produce immunopathology including direct damage to myelin and to oligodendrocytes (Selmaj and Raine 1988). Barnes et al. demonstrated that stimulation of cells with *M. leprae* or lipoarabinomannam (the dominant lipopolysaccharide of *M. leprae*) resulted in high levels of TNF production by cells from tuberculoid leprosy patients, and lower levels from lepromatous patients' cells. However cells from patients with erythema nodosum leprosum produced extremely high levels during the acute phase of the reaction though these levels fell to the lepromatous range once the episode had abated (Sarno et al. 1991). It seems likely that the levels of TNF produced in tuberculoid patients have a useful effect in controlling the disease. However, in patients with type 2 reactions the levels of TNF production are sufficient to produce pathology such as the nerve damage and tissue necrosis (Barnes et al. 1992).

In type 1 leprosy reactions there is an increase in cell mediated immunity towards *M. leprae* that may result in severe pathology in the nerves and skin. Khanolkar-Young et al. have demonstrated high levels of TNFα mRNA and protein in the skin and nerve macrophages of people with reactions (Khanolkar-Young et al. 1995).

Roy et al. have investigated the association of TNF polymorphisms in a population of leprosy patients from Calcutta. They found that the mutant allele of the –308 promoter polymorphism was associated with lepromatous but not tuberculoid leprosy (O.R. 3.0 p=0.02) (Roy et al. 1997).

5.4.5 RESULTS FOR TNF

The results are shown in tables 5.14-5.18. No significant difference was found between - 308 polymorphism frequencies in cases or controls, or between different forms of leprosy. In particular, there was no difference between the patients who developed ENL compared with those lepromatous leprosy cases that had never developed ENL, despite the fact that these individuals are known to have markedly elevated TNF levels during reactions (Sarno et al. 1991). There was, however, a marginally significant difference between cases and controls (p=0.042), with an excess of people homozygous for the common form of the –238 allele amongst the cases. When the largest ethnic group, the Padayachis, were examined, the trend persisted but was no longer significant. A non significant trend in the same direction occurred when allele frequencies alone were looked at.

Disappointingly, there was no evidence of any difference in allele frequencies

TNF -238	Controls	ENLs	LL controls	Total MBs	PBs	Total cases
11	145 (81.5)	80 (88.9)	32 (82.1)	132 (88.6)	156 (87.6)	288 (88.1)
12	31 (17.4)	8 (8.9)	6 (15.4)	14 (9.4)	21 (11.8)	35 (10.7)
22	2 (1.1)	2 (2.2)	1 (2.6)	3 (2)	1 (0.6)	4 (1.2)
Total	178	90	39	149	178	327

Table 5.14 TNF -238 genotypes (% in brackets) (1 represents the wild type allele, 2 the mutant allele)

(MB = multibacillary cases, ENL = erythema nodosum leprosum cases. LL Controls = lepromatous leprosy controls who had not developed ENL after at least 1 year of therapy. Total MB cases include some cases where it was uncertain whether or not they had suffered from or were going to suffer from ENL, hence the discrepancy between ENLs plus LL controls and Total MBs).

2x2χ^2 = 4.12, p=0.042 OR=1.68 {CI= 0.98-2.87} comparing Cases vs. Controls (11 vs. 12+22)

2x2χ^2 =1.11, p=0.292 comparing LL Controls vs. ENLs (11 vs. 12+22)

3x2χ^2 (2d.f.)= 1.84 p=0.398 comparing MB vs. PB cases

Mantel Haenszel test for 2 largest castes χ^2 = 2.55 p=0.110

TNF –238 allele	Controls	ENLs	LL controls	Total MBs	PBs	Total cases
1	321 (90.2)	168 (93.3)	70 (89.7)	278 (93.3)	333 (93.5)	611 (93.4)
2	35 (9.8)	12 (6.7)	8 (10.3)	20 (6.7)	23 (6.5)	43 (6.6)
Total	356	180	78	298	356	654

Table 5.15 TNF -238 allele frequencies (% in brackets)

(MB = multibacillary cases, ENL = erythema nodosum leprosum cases. LL Controls = lepromatous leprosy controls who had not developed ENL after at least 1 year of therapy. Total MB cases include some cases where it was uncertain whether or not they had suffered from or were going to suffer from ENL, hence the discrepancy between ENLs plus LL controls and Total MBs).

Cases vs. Controls $2x2\chi^2 = 3.43$, $p=0.064$

LL Controls vs. ENLs $2x2\chi^2 = 0.98$, $p=0.322$

MB vs. PB cases $2x2\chi^2 = 0.02$ $p=0.897$

TNF –238 genotype	Controls	ENLs	LL controls	Total MBs	PBs	Total cases
11	49 (77.8)	16 (84.2)	1	23 (85.2)	56 (86.2)	79 (85.9)
12	13 (20.6)	2 (10.5)	1	3 (11.1)	9 (13.8)	12 (13.0)
22	1 (1.6)	1 (5.3)	0	1 (3.7)	0	1 (1.1)
Total	63	19	2	27	65	92

Table 5.16 TNF -238 genotypes in Padayachis alone (alleles are 1 and 2)

(MB = multibacillary cases, ENL = erythema nodosum leprosum cases. LL Controls = lepromatous leprosy controls who had not developed ENL after at least 1 year of therapy. Total MB cases include some cases where it was uncertain whether or not they had suffered from or were going to suffer from ENL, hence the discrepancy between ENLs plus LL controls and Total MBs).

Cases vs. controls (11 vs. 12+22) $2x2\chi^2 = 1.70$ p=0.192

MB cases vs. PB cases (11 vs. 12+22) $2x2\chi^2 = 0.01$ p=0.903

TNF –308 genotype	Controls	ENLs	LL controls	Total MBs	PBs	Total cases
11	164 (88.6)	82 (88.2)	39 (92.9)	130 (89.7)	163 (88.1)	293 (88.8)
12	21 (11.4)	11 (11.8)	3 (7.1)	15 (10.3)	21 (11.4)	36 (10.9)
22	0	0	0	0	1 (0.5)	1 (0.3)
Total	185	93	42	145	185	330

Table 5.17 TNF -308 genotypes in South Indian subjects (% in brackets) (alleles are 1 and 2)

(MB = multibacillary cases, ENL = erythema nodosum leprosum cases. LL Controls = lepromatous leprosy controls who had not developed ENL after at least 1 year of therapy. Total MB cases include some cases where it was uncertain whether or not they had suffered from or were going to suffer from ENL, hence the discrepancy between ENLs plus LL controls and Total MBs).

Cases vs. Controls (11 vs. 12+22) $2x2\chi^2 = 0.00$, p=0.962

LL Controls vs. ENLs (11 vs. 12+22) $2x2\chi^2 = 0.68$, p=0.408

MB vs. PB cases $2x2\chi^2 = 0.20$ p=0.658

TNF –308 alleles	Controls	ENLs	LL controls	Total MBs	PBs	Total cases
1	349 (94.3)	175 (94.1)	81 (96.4)	275 (94.8)	347 (93.8)	622 (94.2)
2	21 (5.7)	11 (5.9)	3 (3.6)	15 (5.2)	23 (6.2)	38 (5.8)
Total	370	186	84	290	370	660

Table 5.18 TNF -308 allele frequencies in South Indian subjects(% in brackets)

(MB = multibacillary cases, ENL = erythema nodosum leprosum cases. LL Controls = lepromatous leprosy controls who had not developed ENL after at least 1 year of therapy. Total MB cases include some cases where it was uncertain whether or not they had suffered from or were going to suffer from ENL, hence the discrepancy between ENLs plus LL controls and Total MBs).

Cases vs. Controls $2x2\chi^2 = 0.00$, $p = 0.957$

LL Controls vs. ENLs $2x2\chi^2 = 0.65$, $p = 0.422$

MB vs. PB cases $2x2\chi^2 = 0.33$ $p = 0.568$

between the different types of leprosy or between the cases of lepromatous leprosy that went on to get ENL and those that did not. However, the number of uncomplicated LL cases was very small, and the overall frequency of the 238 mutant allele was also low.

5.4.6 DISCUSSION

It now seems very likely that the certain genotypes of TNF may be beneficial in protection from various infectious diseases. Unfortunately these beneficial effects may have serious pathological side-effects. Apart from an increased predisposition to various chronic inflammatory conditions such as rheumatoid arthritis, patients may also have life-threatening complications from the excessive amounts of TNF produced (Tracey and Cerami 1993). In leprosy these complications manifest themselves as erythema nodosum leprosum, a destructive inflammatory reaction that can result in severe and permanent nerve damage.

The results obtained in this study of a South Indian population are suggestive of a possible association between polymorphisms of the TNF – 238 region and susceptibility to leprosy per se. The significance level is borderline and confirmation of these results is needed in other populations. The -238 polymorphism is not thought to be functional, so these findings may relate to chance alone, although another nearby linked polymorphism could produce the same results. Alternatively, it may be functional after all, possibly affecting the speed and magnitude of the response such that individuals with the –238 wild type allele produce greater tissue damage. If the association were confirmed, further investigations into the biological mechanism would be necessary, but it could lead to new therapeutic approaches. We did not replicate Roy et als.' findings of an excess of mutant

–308 alleles amongst lepromatous cases in our study population (Roy et al. 1997).

It is interesting to note that there was no evidence of linkage with the TNFα microsatellite in the genome screen. This does not exclude a role for TNF though. In the first instance the power of genome screening to detect genes with a small effect is low. Secondly, the role of TNF may be related to specific forms or reactions of leprosy rather than to susceptibility to leprosy per se and would therefore be missed due to the study design. Finally, D6S273 showed some evidence of linkage with leprosy per se in the genome screen. This marker maps closely to TNF. It may be that there are other polymorphisms of TNF that are not in linkage disequilibrium with any of the TNFα microsatellite alleles, but are linked to one of the alleles of D6S273. Alternatively the data may have been skewed by chance or as a result of failure of certain microsatellite markers amplifying better in some families than in others.

5.5 CONCLUDING REMARKS

We have identified a minor association of susceptibility to leprosy per se with the –238 TNF polymorphism which has not been replicated in Roy's population of leprosy patients from Calcutta. Apart from this, the cytokine polymorphisms that we have studied have not provided any clues into the determinants of susceptibility to leprosy, but more polymorphisms will become available in due course and may be worth investigating further. Numerous other cytokines are involved in leprosy. Decisions will need to be made to rationalise the investigation of these as more and more functional polymorphisms are recognised.

We still have a great deal to learn about the complex interactions of the cytokines

and the fine balance that they determine between health and disease. Although leprosy is now curable, there is still a great deal of morbidity attributable to reversal reactions. If we knew who was at high risk of going on to develop leprosy reactions because of genotypic features, efforts could be concentrated on early detection and treatment of these individuals. Understanding the mechanisms involved in the development of reactions might help us to evolve treatments could be to prevent them happening. With its immunological spectrum, leprosy may help provide some more insight into the Th1/Th2 paradigm. This may help us to understand more about infectious, autoimmune and allergic conditions and give clues as to the optimal methods of exploiting this T-cell diversity in the prevention or treatment of disease.

CHAPTER 6

6 CANDIDATE GENE STUDIES PART 2: NRAMP AND VDR

6.1 NRAMP

6.1.1 BACKGROUND

One gene that is a particularly strong candidate for susceptibility to mycobacterial infections in humans is NRAMP1, the human homologue of the mouse gene, Nramp1, the natural resistance associated macrophage protein 1 (also known as Bcg, Ity or Lsh) (Skamene et al. 1982). Nramp1 is situated on mouse chromosome 1 and controls natural resistance to infection with several unrelated intracellular parasites, which include Salmonella, Leishmania and several mycobacterial species (M. bovis, M.avium and *M. lepraemurium*). In 1993 Nramp1 was positionally cloned (Vidal et al. 1993). The gene encodes a 548 amino acid integral membrane protein with 12 transmembrane domains, a glycosylated extracytoplasmic loop, and an intracytoplasmic consensus transport signature that is expressed on macrophages (Bairoch 1993) (fig. 6.1). Susceptibility to mycobacterial infections was associated with a non-conservative glycine to aspartic acid substitution in the fourth transmembrane domain in mice (Vidal et al. 1993) and (Malo et al. 1994). The structure of this protein is shared with ion channels and transporters that are found in a number of prokaryotic and eukaryotic membranes.

It has been found that Nramp1 is specifically located in the late endocytic compartment of macrophages and in the phagosomal membrane after phagocytosis

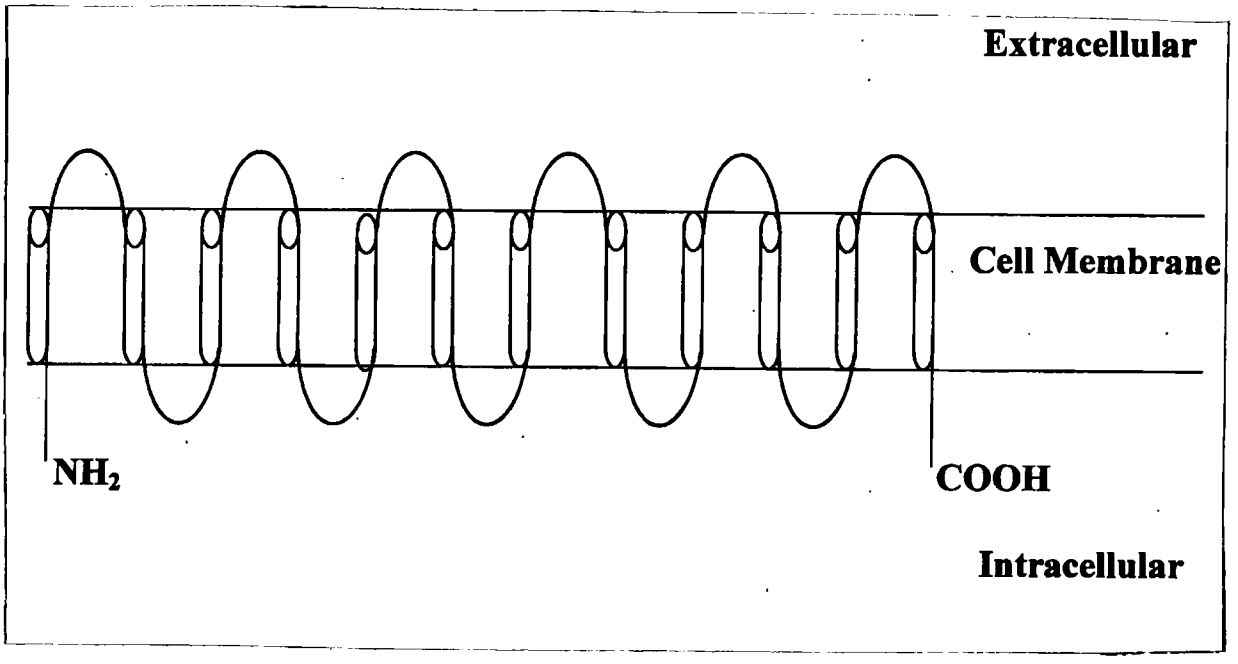

Fig.6.1 The structure of Nramp (based on structure proposed by Vidal et al. Cell 1993)

(Grunheid et al. 1997). Nramp appears to control the replication of intracellular para - sites during the early phase of infection in reticuloendothelial organs. Recently, Hackam et al. demonstrated that there was a significant difference in the acidity of phagosomes containing live mycobacteria in mice with and without the Nramp1 mutation. This was partially due to enhanced pumping of hydrogen ions across the phagosomal membrane and partially to an increased ability of the phagosomes to fuse with endosomes and lysosomes following ingestion of live mycobacteria (Hackam et al. 1998). These findings are consistent with the structure of Nramp1, the actions of similar proteins in other organisms, and the recent findings of the iron transporting function of NRAMP2 (Fleming et al. 1997). NRAMP1 has been mapped to chromosome 2q35.

6.1.2 NRAMP1 POLYMORPHISMS

Nine sequence variants have been identified in the human homologue of Nramp1, NRAMP1 (Liu et al. 1995). White et al have identified a further polymorphic microsatellite in exon 2 (White et al. 1994).

6.1.3 NRAMP AND DISEASE

Several people have speculated that NRAMP1 might be a good candidate for susceptibility to mycobacterial disease in humans (Skamene et al. 1982). It has been implicated in autoimmune disease (Cornall et al. 1991), which may reflect evolutionary influences if it plays a protective role against infectious diseases in man as it does in animals. Shaw et al. looked at 149 individuals with leprosy from 35 multicase families and found no convincing evidence for either a disease susceptibility allele for leprosy per se, or for one controlling the form of disease or response to *M. leprae* soluble antigen. In fact

LOD scores were less than -2, consistently against the possibility of a gene of this type in the region. The families were from two distinct geographical regions and were relatively small in number so these results could have been due to low power and/or ethnic genetic differences. They also looked at NRAMP1 polymorphisms in some tuberculosis families, and these did show some evidence that it might be playing a role in tuberculosis susceptibility (Shaw et al. 1993). In some more recent work Roger et al. looked at the mutations of NRAMP1 found by Liu et al., as well as 3 microsatellite markers in the region, in 7 multicase pedigrees from Polynesia. These included 39 leprosy cases. Their results were again strongly against NRAMP1 being linked to leprosy susceptibility in these pedigrees (Roger et al. 1997). Abel et al. looked at 20 multiplex leprosy families from Vietnam and found that there was segregation of NRAMP1 haplotypes with leprosy though the lod scores were relatively low (Abel et al. 1998).

The most convincing evidence that NRAMP1 does play a significant role in human mycobacterial disease comes from a large case-control study by Bellamy et al. They looked at 4 NRAMP polymorphisms in smear positive tuberculosis cases and controls from Africa. They found that a single nucleotide transversion in intron 4 (469+14G/C) and a TGTG deletion in the 3' untranslated region (55 nucleotides 3' to exon 15), referred to as 1729+55del4, were significantly associated with susceptibility to tuberculosis and were not in linkage disequilibrium. Heterozygotes for both these alleles had a risk of 4.07 (C.I. 1.86-9.12) of developing tuberculosis. In addition a microsatellite polymorphism at -150 base pairs from the start codon was weakly associated with tuberculosis ($p=0.03$) (Bellamy et al. 1998).

6.1.4 RESULTS

The results are shown in tables 6.1 – 6.19. There was no association between the intron 4 polymorphism and either leprosy type or form, or susceptibility to leprosy per se (tables 6.1 and 6.2). There was trend towards an excess of mutant homozygotes in the controls compared with total cases, but this was not significant. Risk of developing a leprosy reaction was unaffected by intron 4 genotype (table 6.3). NRAMP1 microsatellite alleles were present at similar frequencies in multibacillary and paucibacillary cases, and in controls (tables 6.4 and 6.5). When broken down into pure lepromatous (LL) and pure tuberculoid (TT) cases, there appeared to be an excess of individuals with the rarer 201 and 203 alleles amongst the LL cases, but these findings were not significant (table 6.6). Similar findings were seen when risk of leprosy reaction was examined, people experiencing type 1 reactions (those with polar and borderline lepromatous disease) having a non significant excess of the rarer alleles whereas those experiencing type 2 reactions had an excess of the common 199 allele (table 6.7).

There was a significant association between the NRAMP1 1729+55del4 polymorphism and leprosy type in this population (table 6.8). A significant excess of heterozygotes were found amongst the multibacillary cases, and an excess of mutant homozygotes amongst the tuberculoid cases (χ^2 = 8.88, p = 0.003, O.R.= 5.79 with confidence intervals of 1.46 – 24.61). This result persisted after logistic regression was performed to take account of regional, ethnic and sex differences. Mantel Haenszel tests were also performed using the four largest ethnic, and the four largest regional groups to ensure that population stratification was not confounding the results. The p-value remained significant at 0.012 when stratifying for ethnic group, but rose to 0.056 when

Intron 4 Type	MB	PB	Total Cases	Controls	Total
WW	121(84.0%)	64(82.1%)	185(83.3%)	134(80.2%)	319
WM	23(16.0%)	13(16.7%)	36(16.2%)	27(16.2%)	63
MM	0	1(1.3%)	1(0.05%)	6(3.6%)	7
Total	144	78	222	167	389

Table 6.1 NRAMP Intron 4 genotypes in multibacillary (MB) and paucibacillary (PB) cases, and controls from Mali

(*W* = wild allele, *M* = mutant allele)

2x3 χ^2 =1.88 (2d.f.), p=0.390 for MB versus PB leprosy cases

2x3 χ^2 =5.34 (2d.f.), p=0.069 for controls versus all leprosy cases

2x2 χ^2 =4.64 (1d.f.), p=0.031 for controls versus leprosy cases when comparing heterozygotes with mutant homozygotes. By Fisher's exact test (2-tailed) p=0.046. Mantel Haenszel test controlling for 4 largest ethnic groups χ^2 =1.24 p=0.266. Mantel Haenszel test controlling for 4 largest regional groups χ^2 =1.05 p=0.307. The results were not significant after logistic regression taking into account ethnic group, region and sex.

Controls are in Hardy-Weinberg equilibrium

Intron 4 Type	LL	TT
WW	83(83.0%)	33(84.6%)
WM	17(17.0%)	5(12.8%)
MM	0	1(2.6%)
Total	100	39

Table 6.2 NRAMP1 Intron 4 genotypes in cases of lepromatous (LL) and tuberculoid (TT) leprosy in Mali

(*W* = wild allele, *M* = mutant allele)

2x3 χ^2 =2.88 (2d.f.), p=0.237 for LL vs. TT cases

Intron 4 Type	Type 1 reaction	Type 2 reaction	No reaction
WW	25(83.3%)	67(81.7%)	93(84.5%)
WM	5(16.7%)	15(18.3%)	16(14.5%)
MM	0	0	1(0.9%)
Total	30	82	110

Table 6.3 NRAMP1 Intron 4 genotypes in cases with reactions

(*W* = wild allele, *M* = mutant allele)

3x2 χ^2=0.35 (2d.f.), p=0.840 comparing leprosy cases with Type 1 reaction vs. cases without reaction

3x2 χ^2=1.20 (2d.f.), p=0.549 comparing leprosy cases with Type 2 reaction vs. cases without reaction

3x2 χ^2=1.43 (2d.f.), p=0.489 comparing leprosy cases with reaction vs. cases without reaction

Microsatellite Allele	MB	PB	Total Cases (MB + PB)	Controls
199/199	117 (64.6%)	62 (66.7%)	179 (65.3%)	119 (61.0%)
199/201	48 (26.5%)	27 (29.0%)	75 (27.4%)	63 (32.3%)
199/203	5 (2.8%)	3 (3.2%)	8 (2.9%)	1 (0.5%)
201/201	7 (3.9%)	1 (1.1%)	8 (2.9%)	9 (4.6%)
201/203	4 (2.2%)	0	4 (1.5%)	2 (1.0%)
203/203	0	0	0	1 (0.5%)
Total	181	93	274	195

Table 6.4 NRAMP microsatellite genotypes in multibacillary (MB) and paucibacillary (PB) cases, and controls in Mali

Microsatellite Allele	MB	PB	Total Cases	Controls
199	287 (79.3%)	154 (82.8%)	441 (80.5%)	302 (77.4%)
201	66 (18.2%)	29 (15.6%)	95 (17.3%)	83 (21.3%)
203	9 (2.5%)	3 (1.6%)	12 (2.2%)	5 (1.3%)
Total	362	186	548	390

Table 6.5 NRAMP microsatellite allele frequencies in multibacillary (MB) and paucibacillary (PB) cases and controls from Mali

(Alleles are *199*, *201* and *203* base pairs long)

2x3 χ^2 =3.17 (2d.f.), p=0.205 comparing total cases (MB+PB) vs. controls

2x3 χ^2 =1.11 (2d.f.), p=0.574 comparing MB vs. PB cases

Microsatellite Allele	LL	TT
199	198 (77.3%)	74 (82.2%)
201	50 (19.5%)	15 (16.7%)
203	8 (3.1%)	1 (1.1%)
Total	256	90

Table 6.6 NRAMP1 microsatellite allele frequencies in cases of lepromatous (LL) and Tuberculoid (TT) leprosy

(Alleles are *199*, *201* and *203* base pairs long)

$2x3\chi^2 = 1.53$ (2d.f.) p=0.465 for LL vs. TT cases

$2x2\chi^2 = 0.94$ (1d.f.) p=0.332 for LL vs. TT cases comparing allele 199 with others

NRAMP microsatellite allele	Type 1 reaction	Type 2 reaction	No reaction
199	70 (85.4%)	161 (76.7%)	210 (82.0%)
201	11 (13.4%)	42 (20.0%)	42 (16.4%)
203	1 (1.2%)	7 (3.3%)	4 (1.6%)
Total	82	210	256

Table 6.7 NRAMP1 microsatellite allele frequencies in cases of leprosy reactions in Mali

(Alleles are *199*, *201* and *203* base pairs long)

Cases of leprosy with Type 1 reaction vs. cases without reaction 3x2 χ^2=0.49 (2d.f.), p=0.784

Cases of leprosy with Type 2 reaction vs. cases without reaction 3x2 χ^2=2.78 (2d.f.), p=0.250

Cases of leprosy with reaction vs. cases without reaction 3x2 χ^2=1.25 (2d.f.), p=0.536

NRAMPTGTG Type	MB	PB	Controls	Total
TGTG/TGTG	105 (58.0%)	55 (59.8%)	118 (58.7%)	278
TGTG/Del	72 (39.8%)	28 (30.4%)	71 (35.3%)	171
Del/Del	4 (2.2%)	9 (9.8%)	12 (6.0%)	25
Total	181	92	201	474

Table 6.8 NRAMP1 1729+55del4 (NRAMPTGTG) allele types in multibacillary (MB) and paucibacillary (PB) cases, and controls in Mali

(Alleles are *TGTG* {wild type} and *Del* {mutant allele with TGTG deletion})

Overall comparison of multibacillary cases versus paucibacillary cases 3x2 χ^2 = 8.83 (2d.f.), p=0.012

2x2 table of leprosy type vs. heterozygotes/homozygous mutant status, χ^2 =8.88 (1d.f.), p=0.003 Odds ratio = 5.79 (CI 1.46-24.61). 2-tailed Fisher's exact test p=0.009

Comparison of leprosy type vs. heterozygotes/homozygous mutant status cases 2x2 Mantel - Haenszel for the 4 largest ethnic groups:χ^2= 6.29, p=0.012 Odds ratio=6.87 (C.I. 1.44-39.41).

Comparison of leprosy type vs. heterozygotes/homozygous mutant status cases 2x2 Mantel - Haenszel for the 4 largest regional groups:χ^2= 3.64, p=0.056 Odds ratio=4.62 (C.I. 0.99-29.91).

The effect of NRAMP TGTG genotype on leprosy type remained after logistic regression accounting for sex, region and ethnic group (p=0.005).

Controls are in Hardy Weinberg equilibrium

stratifying for regional group. A similar trend was seen when the population was broken down into polar lepromatous and polar tuberculoid cases, with an excess of mutant homozygotes in the tuberculoid group and excess heterozygotes amongst the lepromatous cases (tables 6.9 and 6.10). When a 2x2 χ^2 was performed the p-value was 0.0304, but this disappeared after the Mantel Haenszel test was performed to account for the four largest regional and ethnic groups. A similar study in a South Indian population showed no evidence of an association between the NRAMP1 1729+55del4 polymorphism and leprosy form (table 6.18 and 6.19).

An excess of *TGTG del* alleles were present in people experiencing a type 1 leprosy reaction, the majority of these being accounted for by those with paucibacillary leprosy. The number of lepromatous leprosy patients who experienced type 1 leprosy reactions was low, but the percentage of homozygotes for the mutant allele was much lower in this group than in those with tuberculoid leprosy. This reflects the original association of leprosy form with NRAMP1 1729+55del4 genotype (tables 6.10 and 6.12). All people experiencing type 2 reactions were, by definition, multibacillary cases so it was no surprise that there were a greater percentage of heterozygotes amongst this group (table 6.11). Despite the above trends, there was no significant difference between cases experiencing reactions (type 1 or type 2) and cases that had no reactions.

Table 6.13 shows the breakdown of MB vs. PB cases by ethnic group, with percentages of cases shown for the four largest groups. There were more Bambara cases than controls and fewer Malinke cases than controls, hence the requirement to perform

NRAMP TGTG Type	LL	TT
TGTG/TGTG	70 (54.3%)	26 (57.8%)
TGTG/Del	56 (43.4%)	15 (33.3%)
Del/Del	3 (2.3%)	4 (8.9%)
Total	129	45

Table 6.9 NRAMP1 1729+55del4 (NRAMPTGTG) genotypes in cases of lepromatous (LL) and tuberculoid (TT) leprosy

(Alleles are *TGTG* {wild type} and *Del* {mutant allele with TGTG deletion})

3x2 χ^2=4.48 (2d.f.), p=0.107 comparing leprosy type with genotype

2x2 χ^2=4.49 (1d.f.), p=0.034 Odds ratio = 4.98 (C.I. 0.81-32.38) 2-tailed p-value (Fisher's exact test) = 0.056 comparing leprosy type with heterozygous and homozygous mutant genotypes

2x2 Mantel - Haenszel χ^2 (heterozygotes vs. homozygous mutants in LL vs. TT cases) accounting for the 2 largest ethnic groups =5.13, p=0.113 Odds ratio=5.13 (0.8-48.27)

2x2 Mantel - Haenszelχ^2 (heterozygotes vs. homozygous mutants in LL vs. TT cases) accounting for the 4 largest regional groupsχ^2 =1.22, p=0.269 Odds ratio=3.8 (0.52-38.94).

NRAMP TGTG Type	No reaction	Type 1 reaction	Type 2 reaction
TGTG/TGTG	74 (59.7%)	27 (64.3%)	59 (55.1%)
TGTG/Del	44 (35.5%)	11 (26.2%)	45 (42.1%)
Del/Del	6 (4.8%)	4 (9.5%)	3 (2.8%)
Total	124	42	107

Table 6.10 NRAMP1 1729+55del4 (NRAMPTGTG) genotypes in cases of leprosy reactions in Mali

(Alleles are *TGTG* {wild type} and *Del* {mutant allele with TGTG deletion})

Cases of leprosy with Type 1 reaction vs. cases without reaction 3x2 χ^2=2.07 (2d.f.), p=0.355

Cases of leprosy with Type 2 reaction vs. cases without reaction 3x2 χ^2=1.46 (2d.f.), p=0.482

Cases of leprosy with reaction vs. cases without reaction 3x2 χ^2=0.13 (2d.f.), p=0.938

NRAMP TGTG Type	No reaction & MB leprosy	Type 1 reaction & MB leprosy	Type 2 reaction & MB leprosy
TGTG/TGTG	31 (60.8%)	15 (65.2%)	59 (55.1%)
TGTG/Del	20 (39.2%)	7 (30.4%)	45 (42.1%)
Del/Del	0	1 (4.3%)	3 (2.8%)
Total	51	23	107

Table 6.11 NRAMP1 1729+55del4 (NRAMPTGTG) genotypes in cases of type 1 leprosy reactions broken down into multibacillary cases in Mali

NRAMP TGTG Type	No reaction & PB leprosy	Type 1 reaction & PB leprosy
TGTG/TGTG	43 (58.9%)	12 (63.2%)
TGTG/Del	24 (32.9%)	4 (21.1%)
Del/Del	6 (8.2%)	3 (15.8%)
Total	73	19

Table 6.12 NRAMP1 1729+55del4 (NRAMPTGTG) genotypes in cases of type 1 leprosy reactions broken down into paucibacillary cases in Mali

(Alleles are *TGTG* {wild type} and *Del* {mutant allele with TGTG deletion})

Cases of MB leprosy with Type 1 reaction vs. cases of MB leprosy without reaction 3x2 $\chi^2 = 2.60$ (2d.f.), p=0.272

Cases of MB leprosy with Type 2 reaction vs. cases of MB leprosy without reaction 3x2 $\chi^2 = 1.69$ (2d.f.), p=0.429

Cases of PB leprosy with Type 1 reaction vs. cases of PB leprosy without reaction 3x2 $\chi^2 = 1.62$ (2d.f.), p=0.445

ETHNIC GROUP	Frequency of NRAMPTG TG wild type allele	MB (% cases)	PB (% cases)	TOTAL NO. OF CASES	CONTROLS
Bambara	0.75	79(44%)	39(42%)	118(43%)	71(35%)
Bobo	0.5	1	1	2	1
Bozo/Somono	0.77	5	1	6	3
Dogon	0.82	7	2	9	2
Forgeron	1.00	1	0	1	3
Kassouke	0.8	2	1	3	2
Kissie	1.00	0	0	0	1
Malinke	0.79	16(9%)	11(12%)	27(10%)	37(18%)
Minianka	0.7	0	0	0	5
Moor	0.75	3	0	3	1
Ouolof	1.00	1	0	1	1
Peulh/Foulah	0.73	36(20%)	25(27%)	61(22%)	35(17%)
Sarakole	0.84	18(10%)	7(8%)	25(9%)	24(12%)
Senoufo	0.75	0	1	1	5
Song/rhai	0.77	9	4	13	9
Sonike	0.83	3	0	3	0
Touareg	0.5	0	0	0	1

Table 6.13 Ethnic group distribution, and the frequency of NRAMP1 1729+55del4 (NRAMPTGTG) allele amongst them

(Alleles are *TGTG* {wild type} and *Del* {mutant allele with TGTG deletion})

the Mantel – Haenszel test to eliminate this potential confounding effect. The frequency of the NRAMP TGTG allele is shown for each group and is not markedly different between the groups. Combined analysis of NRAMP1 haplotypes for NRAMP TGTG and the Intron 4 alleles show no evidence of an association between NRAMP1 and leprosy (table 6.14). Tables 6.15 to 6.17 show the relationship between possession of the different alleles of the 3 polymorphism that I looked at. There was evidence of strong linkage disequilibrium between the *199* microsatellite and both the TGTG wild type allele and the Intron4 *G* allele. It is therefore rather surprising that there was no evidence of linkage between the NRAMP TGTG wild type allele and the Intron4 G allele, since the Intron4 region is midway between the microsatellite and NRAMP TGTG. These findings are consistent with those of Bellamy et al. in a closely related Gambian population although they do not comment on any association of the microsatellite with the NRAMP TGTG polymorphism. The association of leprosy form with the NRAMP TGTG polymorphism but not with the *199* microsatellite may indicate that the actual causative polymorphism is not NRAMP TGTG itself, but another polymorphism 3' to it.

In view of the findings with the NRAMP TGTG polymorphism in Mali, we performed similar studies in Tamil subjects (tables 6.18 and 6.19). Paucibacillary (PB) cases comprised of the affected sibling pairs used in the first round of the genome screen. Using the haplotype relative risk method "virtual" controls were derived from the non-inherited parental alleles of these PB cases (see section 1.7.1). Caste matched controls were used in the study of lepromatous and ENL cases. No significant association was found between any of the groups and alleles or genotypes of NRAMP TGTG. The *del* allele was markedly less common in this population, so numbers were

NRAMPTGTG /Intron4 Type	MB	PB	Leprosy Case	Leprosy Control	χ^2 test Yates corrected	p value Yates corrected
TGTG/TGTG /GG	72 (55%)	38 (55%)	110 (53%)	85 (56%)		
TGTG/Del/ GG	44 (34%)	21 (30%)	65 (31%)	41 (27%)	0.49	0.48
TGTG/TGTG /GC	10 (8%)	7 (10%)	17 (8%)	14 (9%)	0	0.98
TGTG/Del/ GC	13 (10%)	4 (6%)	17 (8%)	11 (7%)	0.05	0.82

Table 6.14 NRAMP1 haplotypes in multibacillary (MB) and paucibacillary(PB) cases, and controls from Mali

(Alleles are *TGTG* {wild type} and *Del* {mutant allele with TGTG deletion} for the NRAMP1 1729+55del4 (NRAMPTGTG) polymorphism, and *G* and *C* for the intron4 polymorphism)

	Intron4 GG	Intron4 GC	Intron4 CC	Total
TGTG/TGTG	195	31	3	229
TGTG/Del	106	28	3	137
Del/Del	13	3	1	17
Total	314	62	7	383

Table 6.15 Intron 4 genotype versus NRAMP TGTG genotype in Mali

(Alleles are *TGTG* {wild type} and *Del* {mutant allele with TGTG deletion} for the NRAMP1 1729+55del4 (NRAMPTGTG) polymorphism, and *G* and *C* for the intron4 polymorphism)

$2x3\chi^2$= 5.207 (2d.f.), p=0.267 comparing *GG* and *GC* Intron 4 genotypes with TGTG genotype, suggesting no evidence of linkage between the 2 markers

	199/199	199/201	199/203	201/201	201/203	203/203	Total
TGTG/ TGTG	209(71%)	53 (39%)	4	4	0	0	270
TGTG/ Del	76 (26%)	72 (53%)	5	5	6	1	165
Del/Del	7 (2%)	10 (7%)	0	7	0	0	24
Total	293	136	9	16	6	1	459

Table 6.16 Microsatellite genotype versus NRAMP TGTG genotype in Mali

(Alleles are *TGTG* {wild type} and *Del* {mutant allele with TGTG deletion} for the NRAMP1 1729+55del4 (NRAMPTGTG) polymorphism, and *199*, *201* and *203* for the microsatellite polymorphism)

3x2χ^2 of 199/199 homozygote and 199/201 heterozygotes against NRAMP TGTG genotype: 2x3 $\chi^2 = 41.39$ (2d.f.), p=0.000

3x2 χ^2 of 199/199 homozygote compared to all other microsatellite genotypes against NRAMP TGTG genotype: 2x3 $\chi^2 = 56.46$ (2d.f.), p=0.000. These findings suggest strong linkage between the *199* microsatellite allele and the *TGTG* wild type allele.

	199/199	199/201	199/203	201/201	201/203	203/203	Total
Intron4 GG	239 (99.6%)	64 (56%)	1	6	1	0	311
Intron4 GC	1	44 (39%)	8	6	3	1	63
Intron4 CC	0	6 (5%)	0	0	1	0	7
Total	240	114	9	12	5	1	381

Table 6.17 Microsatellite genotype versus Intron 4 genotype in Mali

(Alleles are *G* and *C* for the Intron4 polymorphism, and *199*, *201* and *203* for the microsatellite polymorphism)

3x2χ^2 of 199/199 homozygote and 199/201 heterozygotes against NRAMP Intron4 genotype: χ^2= 118.3 (2d.f.), p=0.000

3x2χ^2 of 199/199 homozygote compared to all other microsatellite genotypes against NRAMP Intron 4 genotype: χ^2= 139.43 (2d.f.), p=0.000 suggesting strong linkage between the *199* microsatellite allele and the Intron 4 *G* allele

NRAMPTGTG Type	PB	"Virtual Controls"
TGTG/TGTG	145 (84.3%)	146 (84.9%)
TGTG/Del	24 (14.0%)	25 (14.5%)
Del/Del	3 (1.7%)	1 (0.6%)
Total	172	172

Table 6.18 NRAMP1 1729+55del4 (NRAMPTGTG) genotypes in Tamil subjects paucibacillary (PB) cases versus controls (Haplotype relative risk method)

(Alleles are *TGTG* {wild type} and *Del* {mutant allele with TGTG deletion})

Comparison of paucibacillary cases versus "virtual" controls 3x2 χ^2 =1.02 (2d.f.), p=0.599

Controls are in Hardy-Weinberg equilibrium

NRAMPTGTG Type	ENL Cases	Lepromatous leprosy controls	TotalMB cases	Controls
TGTG/TGTG	68 (81.0%)	29 (80.6%)	123 (82.0%)	83 (78.3%)
TGTG/Del	14 (16.7%)	6 (16.7%)	24 (16.0%)	21 (19.8%)
Del/Del	2 (2.4%)	1 (2.8%)	3 (2.0%)	2 (1.9%)
Total	84	36	150	106

Table 6.19 NRAMP1 1729+55del4 (NRAMPTGTG) genotypes in Tamil subjects multibacillary (MB) cases versus controls

(Alleles are *TGTG* {wild type} and *Del* {mutant allele with TGTG deletion})

(N.B. Total MB cases are greater than ENL plus LL controls because other subjects in whom the ENL status was unknown are also included. LL controls are cases of lepromatous leprosy who had not developed ENL after at least 1 year's treatment)

Comparison of multibacillary cases versus controls 3x2 $\chi^2 = 0.62$ (2d.f.), p=0.732

Controls are in Hardy-Weinberg equilibrium

too small to draw any conclusions regarding trends in the same direction as the Mali subjects.

6.1.5 DISCUSSION

NRAMP1 is a very strong candidate for an infectious disease susceptibility gene. The association of the NRAMP TGTG polymorphism with leprosy form is of particular interest in the light of the recent findings of Bellamy et al. They studied a Gambian population (ethnically very similar to Mali), and found that heterozygosity for the NRAMP TGTG polymorphism was associated with sputum positive tuberculosis (Bellamy et al. 1998). Since this form of tuberculosis may be thought of as being analogous to lepromatous leprosy, our findings complement theirs. This contrasts with the findings in mice where only homozygotes of the Nramp1 polymorphism are susceptible to disease. It is of interest that D2S125, which is situated relatively close to the human NRAMP1 gene, was one of the markers in the South Indian leprosy genome screen that was mildly suggestive of linkage. The low lod score would be in keeping with a minor gene effect.

The results were not replicated in the South Indian population (Tables 6.18 and 6.19). One reason for this would be low power in view of the lower frequency of the *del* allele frequency in the Indian population. An alternative explanation is that the causative polymorphism may be in linkage disequilibrium with NRAMP TGTG in Mali but not in all other populations. Finally, the findings in Mali may have been due to chance alone and there may be no association at all. The NRAMP TGTG polymorphism was not found to be linked with leprosy in either Polynesia or Pakistan (Roger et al. 1997) and

(Shaw et al. 1993), although weak evidence for linkage was found in Vietnamese families (Abel et al. 1998).

In mice Nramp1 controls the replication of intracellular parasites during the early phase of infection in reticuloendothelial organs. Possible mechanisms for this are ion transportation or modulation of the intraphagolysosomal pH (Searle et al. 1998) and (Hackam et al. 1998). Heterozygosity for NRAMP TGTG may correlate with optimal intraphagolysosomal conditions for the growth of *M.leprae*. It has recently been found that immunisation of mice with recombinant salmonella raises a predominantly Th1 type response in those with the Nramp1 resistance allele, and a Th2 type response in Nramp1 mutant homozygotes (Soo et al. 1998). The mechanism for this is unknown, but the parallel with NRAMP1 and the different forms of leprosy (which are associated with different immune responses) is intriguing. However, the role that the NRAMP TGTG allele plays is still unknown, and the possibility remains that it is in linkage disequilibrium with another functional polymorphism within NRAMP1, or even in another gene, remains.

Further studies need to be performed to clarify the role of NRAMP1 in determining leprosy form. If a role is suggested by future results, the cellular mechanisms for this will need to be determined. Gene therapy or alternative approaches to treatment may then become possible. Studies on infected human cells of different genotypes could give further insights as will continuation of the already vast amount of animal work.

6.2 VITAMIN D AND LEPROSY

6.2.1 INTRODUCTION

Classically, 1α - 25 dihydroxy vitamin D_3 (1,25 $(OH)_2 D_3$), the active metabolite of vitamin D, is known to be involved in calcium and phosphate homeostasis and in bone mineralisation. Over the last 15 years or so, it has become apparent that vitamin D also has an immunomodulatory role, being involved in the control of both cellular and humoral immunity. Monocytes and macrophages are upregulated by dihydroxy-cholecalciferol, whereas lymphocyte function is depressed. Immunoglobulin production falls, TH1-type helper cell function is reduced with decreased production of IL-2, IL-12 and IFNγ, and there is reduced Class II antigen expression by lymphocytes and monocytes (Lemire et al. 1995), (Rigby et al. 1990), (D'Ambrosio et al. 1998) and (Hustmyer et al. 1992). Cell mediated immunity is enhanced (Hernandez-Frontera and McMurray 1993) and (McMurray et al. 1990). Macrophages and other cells of the immune system can produce $1,25(OH)_2D_3$, which acts in a paracrine manner, controlling cellular and humoral immunity locally. This has the effect of enhancing adherence, chemotaxis, and phagocytosis as well as increasing the levels of H_2O_2, oxygen radicals and heat shock proteins (Casteels et al. 1995) and (Yamamoto and Naraparaju 1996).

The vitamin D receptor (VDR) is required for normal calcium absorption from the gut. It forms homo or hetero dimers with other members of the steroid hormone receptor superfamily and is encoded by a gene on 12q13-14 (Carlberg et al. 1993). Vitamin D receptors are present on cells of the immune system (monocytes and activated T and B lymphocytes) as well as the classical tissues, kidney, gut and liver (Provvedini et al. 1983). The immunoregulatory effects described above are mediated

through the VDR.

6.2.2 VDR POLYMORPHISMS

Morrison et al. defined a number of polymorphisms in the 3' untranslated region of the VDR gene. A Taq1 RFLP polymorphism (T to C substitution conservative for isoleucine) at codon 352 of the vitamin D receptor gene has been associated with bone density and susceptibility to osteoporosis (p=0.0008) (Morrison et al. 1994). (The alleles at this site are designated T and t). The other polymorphisms are in linkage disequilibrium with the Taq1 site such that people either have the baT or Bat haplotypes. There is evidence from transfection studies that the Bat haplotype is associated with increased expression of VDR mRNA (Morrison et al. 1994). This could lead to greater monocyte activation in people with the Bat haplotype. Several further studies investigating the association of the Taq polymorphism with osteoporosis have produced variable results, about half of them confirming Morrison's findings, the others showing no evidence of an association. It is likely that this reflects differences in other genetic and environmetal factors (Ferrari et al. 1998).

6.2.3 VITAMIN D AND DISEASE

Apart from the association with bone mineral density in some populations, vitamin D has been implicated in a variety of diseases. These include autoimmune diseases in experimental animals e.g. prevention of SLE, insulitis, diabetes and experimental allergic encephalitis in mice (Lemire et al. 1995) (Casteels et al. 1995) and (Mathieu et al. 1995). More recently the Taq1 polymorphism has been associated with risk of developing prostate cancer in humans. Men homozygous for the t allele have 1/3 of the risk of

developing prostate cancer compared with men who are heterozygous or homozygous for the T allele (Taylor et al. 1996). $1,25(OH)_2D_3$ has been implicated in defence against infectious diseases and in particular against tuberculosis, details of which are described below. Children with rickets were noted to be at increased risk of infections and 6 weeks of supplementary fish oil resulted in decreased susceptibility to infection with Klebsiella pneumonia and cerebral malaria in C57B1/6 mice (Yener et al. 1995) and (Blok et al. 1992). Prostitutes from Uganda who were homozygous for the tt allele of VDR were more susceptible to HIV, the effect being greater in females (S.Ali, personal communication).

Denis found that $1,25(OH)_2D_3$ resulted in decreased intramacrophage growth of mycobacterium tuberculosis and that there was evidence for significant mycobacterial killing when monocytes were pulsed with $1,25(OH)_2D_3$, IFNγ and TNFα (Denis 1991). There have been numerous reports suggesting further possible links between vitamin D and tuberculosis. These included the clinical effect of calciferol on lupus vulgaris, the fact that tuberculosis is 2.8 times more common in strict vegetarians, and the increased rate of tuberculosis in new immigrants from the Indian subcontinent (Dowling and Prosser-Thomas 1946) and (Strachan et al. 1995). These lead Bellamy et al. to look at vitamin D receptor polymorphisms in tuberculosis. They found that there was a significantly lower risk of developing tuberculosis (O.R.= 0.31 95% C.I. 0.14 - 0.67) in those who were homozygous for the lower bone mineral density allele (tt) compared with other genotypes (Tt and TT) in a Gambian population. In the same study, Bellamy et al. were able to examine susceptibility to clinical malaria and hepatitis B carriage rates with VDR genotypes in the Gambians. Clinical malaria was not associated with VDR. However, for

those individuals with evidence of previous hepatitis B infection, possession of the tt genotype protected them from becoming persistent hepatitis B carriers. Despite linkage disequilibrium between t and A, the AA VDR genotype was independently associated with an increased risk of carrier status in this population (Bellamy et al in press).

In contrast to mycobacterium tuberculosis, there is scanty literature on the effects of vitamin D on leprosy. In 35 villages studied in South India the prevalence of leprosy correlated with malnutrition in children aged 1-4years of age but not in adults (Sommerfelt et al. 1985). Cappuro and Guillot used intramuscular injections of vitamin D in tuberculoid leprosy patients with reactions and found an improvement in the reactional state within 7-21 days. Unfortunately they had no control group (Cappuro and Guillot 1948). Underhill et al. found that leprosy patients retained supplemental calcium whilst normal patients secreted it, although others have found that serum calcium levels were significantly lower in lepromatous leprosy patients than normal controls (Underhill et al. 1920) and (Nigan et al. 1979). There do not appear to be any animal studies looking directly at vitamin D in leprosy but rats fed on a calcium deficient diet had a greater rate of growth of *M. lepraemurium* than controls rats (Badger et al. 1940).

6.2.4 RESULTS

The results of the studies of the Taq 1 polymorphism of the vitamin D receptor in leprosy cases and controls from Mali and from South India are shown in tables 6.20 to 6.23. There was a minor excess of tt homozygotes and slightly fewer heterozygotes amongst the controls when compared with the cases in Mali. No significant difference in the distribution of the different genotypes was found between cases and controls, or

Genotype	Controls	Cases	MB	PB	Total
TT	86 (45.0%)	122 (46.0%)	81 (47.1%)	41 (44.1%)	208 (45.6%)
Tt	71 (37.2%)	104 (39.2%)	67 (39.0%)	37 (39.8%)	175 (38.4%)
tt	34 (17.8%)	39 (14.7%)	24 (14.0%)	15 (16.1%)	73 (16.0%)
Total	191	265	172	93	456

Table 6.20 Taq 1 VDR genotypes in Multibacillary (MB) and Paucibacillary (PB) cases and controls from Mali

(T = wild type allele and t = mutant allele of Taq 1 polymorphism of Vitamin D)

2x3 comparison of cases vs. controls χ^2=0.81 (2df), p=0.667

2x3 comparison of MB vs. PB χ^2=0.32 (2df), p=0.851 (2df)

Genotype	LL	TT
TT	54 (44.3%)	22 (48.9%)
Tt	51 (41.8%)	18 (40.0%)
tt	17 (13.9%)	5 (11.1%)
Total	122	45

Table 6.21 Taq 1 VDR genotypes in lepromatous (LL) and tuberculoid (TT) cases from Mali

(T = wild type allele and t = mutant allele of Taq 1 polymorphism of Vitamin D)

2x3 comparison of LL vs. TT χ^2=0.38 (2df), p=0.827

Genotype	Type 1 reaction	Type 2 reaction	No reaction
11	22 (56.4%)	46 (44.7%)	54 (43.9%)
12	10 (25.6%)	41 (39.8%)	53 (43.1%)
22	7 (17.9%)	16 (15.5%)	16 (13.0%)
Total	39	103	123

Table 6.22 Taq 1VDR genotypes in cases of leprosy with reactions compared to controls

(T = wild type allele and t = mutant allele of Taq 1 polymorphism of Vitamin D)

2x3 comparison of Type 1 reactions vs. no reaction χ^2=3.81 (2df), p=0.148

2x3 comparison of Type 2 reactions vs. no reaction χ=0.41 (2df), p=0.817

2x3 comparison of controls vs. Type 1 and Type 2 reactions χ=1.55 (2df), p=0.461

Genotype	"Virtual" Controls for TT patients	TT Cases	Controls	LL Cases
TT	76(41.3%)	89(48.4%)	30(39.5%)	43(46.7%)
Tt	87(47.3%)	78(42.4%)	34(44.7%)	35(38.0%)
tt	21(11.4%)	17(9.2%)	12(15.8%)	14(15.2%)
Total	184	184	76	92

Table 6.23 Taq 1 VDR genotypes for South Indian Leprosy patients

Haplotype Relative Risk Method used to derive "Virtual Controls" of Tuberculoid (TT) Leprosy Cases (see section 1.8.1).

Standard Case - Control Method used for Lepromatous Leprosy Cases (LL)

(T = wild type allele and t = mutant allele of Taq 1 polymorphism of Vitamin D)

2x3 comparison of TT Cases vs. Controls χ^2=1.94 (2df), p=0.380

2x3 comparison of LL Cases vs. Controls χ^2=0.97 (2df), p=0.616

2x3 comparison of LL vs. TT Cases χ^2=2.27 (2df), p=0.322

2x3 comparison of Controls for TT cases vs. Controls for LL cases χ^2=0.93 (2df), p=0.628

between the different forms or types of leprosy in the Malian subjects. The different types of leprosy reactions were studied. Numbers were relatively small, especially amongst the people with type 1 reactions. Hence, although there appeared to be an excess of both TT and tt homozygotes amongst those experiencing type 1 reactions over both those experiencing type 2 reactions and those who did not experience a reaction, this finding was not significant. It would be hard to find a biologically plausible explanation for this phenomenon.

A similar study was performed in the South Indian patients. Tuberculoid cases comprised of patients being used in the genome screen. Controls for these cases were derived from the parents using the haplotype relative risk method (see section 1.7.1). Lepromatous cases were made up of the ENL cases, the LL controls who had not gone on to develop ENL, and miscellaneous extras. Their controls were matched for caste. Amongst the tuberculoid cases there was a trend towards an excess of individuals posessing the TT genotype, but the findings were not significant. These findings are in the reverse direction to those of Roy et al. In conclusion, no association was found between the Taq1 polymorphism of VDR and leprosy in either the South Indian or the Malian population.

6.2.5 DISCUSSION

At the same time that I performed his study, my colleague, Sushismita Roy performed an identical study on her leprosy cases and controls from Calcutta and found very different results. The tt genotype was over-represented in the tuberculoid leprosy cases (odds ratio 3.22 95%C.I. 1.47-7.13) and the TT genotype was over-represented

amongst lepromatous leprosy cases (odds ratio 1.67 95%C.I. 1.02-2.75) compared to controls (Roy et al 1999).

I did not replicate the results found by Roy et al. in either the S.Indian or the Malian patients. This may be due to either genetic or environmental differences between the two groups (e.g. differences in exposure to sunlight, dietary factors such as calcium intake), or may reflect the fact that the results in the Calcutta population were a chance occurrence. Alternatively, one of these findings may be spurious or there may be alternative genes that control susceptibility that are in linkage disequilibrium with this gene in one population, but not in the other. Because of the known functions of VDR, and the results found in both the Gambian tuberculosis patients, it would be interesting to investigate the *In vitro* function of macrophages of the different genotypes.

The present pattern of VDR genotypes, and consequent prevalence of osteoporosis may well reflect evolutionary processes. The newly discovered role of the VDR in susceptibility to various infectious diseases lends support to this theory.

CHAPTER 7

7 CONCLUDING REMARKS

Unravelling the complex factors involved in determining susceptibility to leprosy per se, and to its different forms, is no simple task. In the past people have mainly concentrated on looking at the HLA genes, but evidence exists that these genes do not account for all the differences in susceptibility between individuals (Risch 1987). With recent advances in the field of genetics, including the Human Genome Project and more sophisticated technology, the possibility of solving this seemingly Herculean problem is becoming a reality.

We have tackled the question with a two-pronged approach. We looked at some candidate genes with known polymorphisms that are potentially functional using a case-control methods. Although more powerful in detecting genes than genome screening, and thus useful for the detection of minor genes, this method relies on a degree of educated guess-work combined with luck as to which polymorphisms have been identified at the time of the study. If associations are identified and subsequently confirmed, our understanding of the mechanisms involved in determining susceptibility to infection may be increased leading to novel therapeutic approaches.

We found an association between leprosy type and an NRAMP1 polymorphism, and a suggestion of an association between susceptibility to leprosy per se and a TNF promoter polymorphism. The NRAMP1 result was found in a population from Mali but was not observed in South India. In view of very convincing findings in tuberculosis

patients (Bellamy et al. 1998), it would be worthwhile investigating the relationship of this polymorphism to leprosy in further populations. The TNF finding was a very borderline association with a polymorphism that has not yet been demonstrated to be functional. Previous studies in a population of leprosy patients from Calcutta had not shown an association between this polymorphism and leprosy form (Roy et al. 1997). TNF remains an interesting candidate gene especially as a determinant of leprosy reactions, and it may be that the – 238 polymorphism is in linkage disequilibrium with another more important polymorphism.

Based upon results of complex segregation analysis which suggest a major gene effect in susceptibility to leprosy (Abel and Demenais 1988), we decided to perform a genome-wide search for leprosy susceptibility loci. This is the largest genome screen to be performed to date in an infectious disease. The potential advantage of this method is that new genes may be identified that might have been overlooked in a candidate gene approach. Simulation studies suggest that the power of a study of this size to detect a major gene is greater than 99% (Davies et al. 1994).

Until just now we had no clear-cut evidence for a major gene effect in susceptibility to leprosy in South India. No marker stood out prominently after the first round screen, and even after the second screen there were no lod scores over 1.47 using either single or multi-point analysis. However, D2S125 and D10S197 looked interesting and Ruby Siddiqui has just performed further genotyping on these two microsatellites using flanking markers. 100 families from Vishakapatnam were used in addition to the 188 Tamil families and there was strong evidence for a genetic effect in the region of D10S197 when the results from all the families were pooled (lod score = 3.006 using

Mapmaker Sibs™). Whether this constitutes a "major gene" effect is debatable, as the effect was only prominent once 288 families had been analysed. Interestingly enough, D10S197 only just scraped through into the second screen. These results are particularly exciting in view of the presence of the macrophage mannose receptor in the close vicinity of this microsatellite. Transmission disequilibrium studies are planned in the near future.

The possibility that there is no "major gene" in leprosy in this South Indian population may be explained in a number of ways. The original complex segregation analyses were performed in the Caribbean. None have been performed in India, and it may be that the two populations have different susceptibility genes. Population heterogeneity within the Tamils, possibly due to caste differences, is another explanation. It is possible that the original results in Desirade were wrong. Segregation analysis relies upon making a number of assumptions. The technique works well for simple Mendelian traits, but in the case of infectious diseases a particular problem arises in modelling for environmental transmission. The finding of a recessive gene for attendance at medical school provides a delightful example of the fact that a degree of scepticism should be exercised in the interpretation of segregation analyses (McGuffin and Huckle 1990).

The leprosy genome screen was originally designed to detect major genes for "leprosy per se", but it turned out that the majority of our sibling pairs had tuberculoid leprosy. It may be that there are different genes that control susceptibility to lepromatous leprosy. In order to be certain that there are no major gene effects in different populations, studies are underway to examine a large cohort of patients from Malawi for leprosy susceptibility genes using an identical approach. It will be interesting to see

whether the findings with D10S197 are repeatable, and whether it is possible to sub-divide the sibling pairs for leprosy type.

No major gene has been mapped for any common infectious disease to date and it may be that evolution has simply selected a large number of minor genes. Several markers on chromosomes 2, 6, 7, 16 and 17 stood out as potential minor genes in different stages of the leprosy genome screen in Tamil Nadu. There was evidence of a minor effect at D6S273, which is in the MHC. This was most prominent on combining the results of the first and second screens. This was a smaller effect than might have been anticipated from the work of Risch et al. (Risch 1987). They calculated that the λ_s attributable to the MHC in tuberculoid leprosy in South India was 1.485 out of a total λ_s of 2.38. It is noteworthy that the results of association studies on the MHC have produced such a varied picture and it is likely that population heterogeneity contributes significantly towards this (Todd et al. 1990) and (Serjeantson 1983).

The X chromosome is another area that we thought might play a role in susceptibility to leprosy. It has long been noted that there are an excess number of men who contract the disease that does not seem to be attributable to ascertainment bias. This pattern is also seen in other infectious diseases and it was interesting that Bellamy et al. found evidence of a genetic effect on the X chromosome in a genome screen of sputum positive tuberculosis cases from The Gambia and South Africa (R. Bellamy - personal communication). Another interesting finding has been the excess of red-green colour-blind individuals amongst leprosy patients, which could again point towards an X-linked gene being involved in leprosy susceptibility (Shwe 1992). There is a red-green colour-blindness gene on Xq28, about 25cM terminal to the X marker identified by Bellamy et

al. The lack of evidence for linkage to this region in our genome screen may be a result of uneven spread of the markers with a largish gap in the region of the colour - blindness gene on Xq28. We did identify a marker that was closer to this region, DXS1766, but found that the microsatellite was non-polymorphic in our population. It may be worthwhile keeping an eye out for other microsatellites in this region and performing further analyses in the future. If there were a major gene on the X chromosome one might have anticipated finding it on a genome screen of this size, but the power of studies on the X chromosome are lower than those on autosomes because full information can only be gleaned when there are sister-sister pairs.

Apart from potential candidate genes identified in the genome screen, there are numerous others that might be worth investigating for more minor effects. Although it's unlikely that the gross defects seen in the IFNγ receptor of the children studied by Newport et al. are also involved in patients with leprosy, it may be that minor defects of this gene are involved (Newport et al. 1996). The fact that IFNγ has been clearly demonstrated to be essential for effective macrophage activity and granuloma formation raises the possibility that mutations within the gene might be involved in lepromatous leprosy where granuloma formation does not occur. Inducible nitric oxide synthase (INOS) is another gene that would be worth investigating further because of the important role that nitric oxide plays in the control of mycobacterial infections. There are numerous cytokine genes that are involved in the complex interaction of the host response to infection, many of which may have polymorphisms and would be worth considering studying. New, less labour intensive, approaches to gene identification are on the horizon. Genome scanning by association should be possible. By present methods it

would involve a lot of work, but has much greater power than linkage methods (Risch and Merikangas 1996). Sibling pairs are not required for this method, which makes collection of cases a great deal more straightforward.

In summary, by using two methods in a search for leprosy susceptibility genes, we have identified a region on chromosome 10 that looks very promising in terms of playing at least a moderate role in leprosy susceptibility in a South Indian population. Several other regions with potential minor genes have also been identified. The samples collected during my two visits to India provide a vast resource that can be used for further studies on susceptibility to leprosy per se, leprosy type and form, and leprosy reactions. The samples from Mali and Calcutta provide us with the opportunity of seeing whether genes identified in South India are responsible for susceptibility to leprosy in other ethnic groups.

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APPENDICES

APPENDIX 1- BREAKDOWN OF WORKLOAD IN THIS THESIS

1) SAMPLES FROM TAMIL NADU

Collection of blood samples and DNA extraction: SJM and KB

Database preparation & examination of clinical notes: SJM

Measurement of DNA concentrations: SJM

Preparation of DNA plates for 1st and 2nd round of genome screen: SJM

Decision of which primers to include in sets 15-20: RS

Genome Screen:

Set	Optimisa-tions	Large-scale PCR	Gel-loading	Genotyping
1	SJM	SJM	SJM	SJM
2	RS	RS	RS	RS
3	SJM	SJM	SJM	SJM
4	RS	RS	RS	RS
5	SEF	SEF/SJM	SJM	SJM
6	SEF	SEF/SJM	SEF/SJM	SEF
7	SEF/SJM	SEF/SJM	SEF/SJM	SEF/SJM
8	SEF	SEF	SJM	SJM
9	SJM	SJM	SJM	SJM
10	SJM	SJM	SJM	SJM
11	SEF	SEF	SEF	SEF
12	RS	RS	RS	RS
13	SJM/RS	SJM/RS	SJM/RS	SJM/RS
14	SJM	SJM	SJM	SJM
15	SJM	SJM	SJM	SJM
16	RS	RS	RS	RS
17	SJM	SJM	SJM	SJM
18	RS	RS	RS	RS
19	SJM	SJM	SJM	SJM
20	SJM	SJM	SJM	SJM
A	RS	RS	SJM	RS
B	RS	RS	RS/SJM	SJM

Global binning and sib-pair analysis was mainly done by chromosome rather than sets. SJM globally binned markers in sets 15, 17, 19, 20 and B, and markers in chromosomes 1, 3, 5 and 7 (95 markers). RS binned the remainder of the markers and

pooled the results from the first and second screens.

2) SAMPLES FROM VISHAKAPATNAM

Collection of blood samples and DNA extraction: SG and team from Agra (minimal help from SJM)

Screening of key markers: RS and KY

3)CANDIDATE GENE WORK ON INDIAN SAMPLES

a) IL4: SJM and SR

b) TNF: SJM (using some samples collected by RMP)

c) VDR: SJM

d) NRAMP: SJM

4)CANDIDATE GENE WORK ON MALI SAMPLES

Collection of blood samples: SM and GW

Database preparation & examination of clinical notes: SM, GW & SS

DNA extraction: SJM, GW and SM

Candidate gene work:

a) VDR: GW and SJM

b) NRAMP: SJM

c) IL-10: SJM

Abbreviations

GW= Giles Warner, KB = K. Balakrishnan, KY = Karen Young, RMP= Prof. R.M. Pitchappan, RS = Ruby Siddiqui, SEF= Simon Fisher, SG = Satish Ghei, SM= Stuart Mucklow, SJM= Sarah Meisner, SR= Suschismita Roy, SS= Samba Sow

APPENDIX 2 - TABLE OF MICROSATELLITE PRIMERS USED

Table shows microsatellite name, primer sequences, fluorescent label and PCR conditions (see Table 2.6 for explanation of PCR conditions)

Marker	Set	Primer sequence (labelled)	Primer sequence (unlabelled)	Label	Conditions
d1s228	1,15	ACT GCA ACA TTG AAA TGG CA	TAG TAT TTT ACA GGG ACC ATA G	HEX	53/2
d1s470	6	AAA TGG GGC TAA TAA ATC TCC TGG	CTA CAC CAT AGG CAT AGG TGC AA	TET	53/2
d1s255	1	TTA GCA AAT CCC AAG CAA TA	GTG ATG GTG GTA AAG GCA GA	HEX	53/2
d1s197	1	TCA TGT CCC TCC TCC CAA AG	GAG CAA GCA TCC AAA AAC GA	FAM	53/2.5
d1s209	1	AAA CAT CAG TGT TCC ATC ATA GAC	AGC TTT GGG GGA CAT AAC AT	HEX	53/2
d1s216	1,17	AGC TTG CAC ACA CCC TAT TC	TCA CAT AAT CCT TCT AAT GTG CT	HEX	51XL/2
d1s207	1	CAC TTC TCC TTG AAT CGC TT	GCA AGT CCT GTT CCA AGT CT	FAM	58/2
d1s252	1,17	AGC TTT TTA CTC TTA ACC TAT TCA T	GCA GAG AAC ATG TGT ATT AAT GA	HEX	51/3
d1s196	1,17	ACC AGC AGC CAA CAT ATG	TTT ACA TTC TCA CAG ACT GTC A	HEX	49XL/2
d1s238	1	GAT CTG CAT TCC AAG ACT CAT G	CAT GGA GGC AGT TTA GAT TGT G	FAM	53L/2
d1s249	15	AAT AAC TCC CCC ATT ATT CAG G	AAT ATG GTT GTA GAT GAG ACT GGC	FAM	58/3
IL-10	17	GAA GTC CTG ATG TCA CTG CC	CAG GAT TCC ATG GAG GCT GG	FAM	53/1
d1s229	1	GCT TGT TTC CAT TTA TGG TG	ACT CTA GTT GTG TGT GAA TGT ATG	HEX	53/2.5
d1s103	1	ACG AAC ATT CTA CAA GTT AC	TTT CAG AGA AAC TGA CCT GT	FAM	55/3
d2s281	14	TGC ATG TGT GTT TGT ATA TGT ATT A	CAT CGT CTT GCA TTA GAC AG	FAM	55/2
d2s131	14	CAA TTG TAC CCC ATT TAC TGC	GGG TCC AAG GAA CTC TCC	HEX	58/3
d2s165	2	GTA ATG CTG TCT CTG AAT CAG G	AGC TTC AAC NCC TTT GAG AT	FAM	51/3
d2s177	2,16	CTT TTT TGA AGG GAA GTG G	TTA CAG GTG TGA GCC ACC	FAM	58/3
d2s134	2	CGT CTG CTC GTC AGA GTC	AGC TCT CCA CGT TTG CTT	FAM	58/2
d2s139	2	ATG TAC CCC ACC CTC AAC	AAC AAG GTT TTC TAA AGT TTC C	FAM	58/3
IL-1a	17	GCC TAG TGA GTG TGG AAG ACA TTG	CAG CAC TGG TTG GTC TTC ATC TTG	FAM	55/3
d2s160	2	TGT ACC TAA GCC CAC CCT TTA GAG C	TGG CCT CCA GAA ACC TCC AA	FAM	53/3
d2s114	2	TTT TTC ATG AAC TGG TAC ACT G	TAG TAC ATT AAC TTG GGT TCC A	HEX	58/1.5
d2s142	2	GGA GAC ACT GAA CGG GTA GA	AGA TTA TCC TAG ATT ACC CAG GG	HEX	58/2
d2s326	2	CAC TTG CTT TTG GAA AAG G	ACC AAG TCA TTG TGT GGA AG	HEX	51/2.5
d2s152	2	CCA AAA ATG TCA TTA TTG TGA	CTC CTT AAG ANC TTG TTT TG	FAM	45/2.5
d3s11	10	CAA ACT TTC CAC AGT ATC GTT C	GTT TCC TTG AGA AGA ATG GAG C	FAM	53/2
d2s72	2	AGC TAT AAT TGC ATC ATT GCA	TGG TCT ATA ACT GGT CTA TG	HEX	53/2.5
d2s157	2	CTG NTA CCC AAA ACT TGG C	GCC TAN TTG ATC GTA GTG AAT T	FAM	58/3

Marker	Set	Primer sequence (labelled)	Primer sequence (unlabelled)	Label	Conditions
NRAMP	19	TTC CGC ATT AGG GCA ACG AG	TTC TGT GCC TCC CAA GTT AGC	TET	55/2
d2s1471	16	Unavailable	Unavailable	FAM	48/3
d2s126	2	AGC ACA GAG CCT ACT TAA TAG C	GTA GCA CAA TCT TAC TAT GCC A	HEX	58/2
d2s159	14	AGG GAT TGG GGA CTG G	GCA GTT TGA GTT TAG GAT GA	FAM	53/3
d2s206	2	TTA AAA ATT AAG TAG GCT TTT GGT T	GTC CTC ATG TGT TTA TGC TGT	HEX	51/2
d2s125	2	GGA GGA TCA CTT GAG CCT	AGC GTT AGA TAC ATT TCA AAA TG	HEX	53/2
d3s1297	3	TTT TTA AGA AGC CCT TCC TGG	GGA AAC ACC TAA TGG GCA AG	FAM	53/2
d3s1560	14	GCA TCT ACA GGG GGT GTC T	AGG CTG ATT TTC AGC ACA A	HEX	55XL/3
d3s1263	3	CTG TTG ACC CAT TGA TAC CC	TAA AAT CAC AGC AGG GGT TC	HEX	58/2.5
d3s1289	17	AAAGCAACTTGTAAGAGAGCA	CTCCTAGATATAATCACTGGCA	TET	51XL/2
d3s1300	3	AGC TCA CAT TCT AGT CAG CC	GAT TGT GCT CAA ATG TCA CA	HEX	53/2
d3s1261	3,19	GAG GTG ATG TGA GAG TGG AT	CTC AGC ACC ACA GTA TGT GT	HEX	55/3
d3s1271	3	AAG GTC CAA GAG TGT GGG G	GCA GAG AGA GGG AGG AAA CA	FAM	53/2
d3s1303	3	CAG ACA ATG GCT TCC AAA AGT A	CAA ACT TAG GGT TGTTCC TCA C	HEX	53/2
d3s1576	14	GCT TTG GAC GCA GGA AGA TA	AGA ACA AAA AGG GAA GAA CCC	HEX	58/2
d3s1279	3	CAC CAT CTG TGT GGT ATT GG	GAC CTA TTT TGG TTA ACA ATT TAG A	HEX	53/2.5
d3s1282	3	CTG AGA TTA AGT TTA TTG GGG	AAT ATA ATC ATG CTA TCT CCA A	FAM	53/2
d3s1262	3	CGG CCC TAG GAT ATT TTC AA	CCA GTT TTT ATG GAC GGG GT	FAM	53/2
d3s1314	14	GAC TCT GCT TCA GGG AAA ACC	CTT CCA GCC CTG AAA AAC AT	HEX	58/2
d3s1265	3	TTC TAT AAG GGC AGG GAC AC	GCT CGC AAT TTC TCC TTA AT	HEX	53/2.5
d4s412	4	ACT ACC GCC AGG CAC T	CTA AGA TAT GAA AAC CTA AGG GA	HEX	53/2
d4s403	4	CAC ACC CAG CAC ACA TCT TT	CCT CTG AAA CAC TAG TAA TTC G	HEX	53/2
d4s418	4	TTG GAG GTG GGG TCT AAT G	ATC ACA GAG TGA AGN AGG ATG	FAM	53/2
d4s405	4	ATC AGG AGA TGT TGC CTT GC	CAG GGC TAT GAT TGG ATG TC	HEX	55/3
d4s398	4	CAT GAA ATG CTG ACT GGG TA	TCA ATT TAT GTG CAG CCA AT	HEX	53/2
d4s231	4,16	AGA TGA GTA TGT TAT TAT ACC	TGC TAG AGT TCC CTA GTG	HEX	48/3
d4s250	4	TGG ACT TGA ACT AGT TCT CCA GC	AGG TTC TCC AGA GAA ACA AAA CC	HEX	53/3
d4s406	4	CTG GTT TTA AGG CAT GTG TG	TCC TCA GGG AGG TCT AAT CA	FAM	53/3
d4s430	4	TAA CCC TGT ATA TGT TAA TGT GC	TAA CCC TGT ATA TGT TAA TGT GC	FAM	couldn't optimise
d4s1565	14	TGC AAA CTG TCA CTC AAA AG	GCC AAG GCT GAT CCT C	HEX	58/3
d4s413	4,17	GAA TAT AGT GCT CCA GAA ACA	GCT ATG GAA TAA TAC CGA GA	FAM	55/2
d4s415	4	GGG CTA AGG CAA CTC C	GGT TAG ATT AAC TGC AAA ACG	FAM	53/2
d4s408	4	CAT GAA AAT TTG AGG GGT CTG	TCC TCA GGG AGG TCT AAT CA	FAM	53/2
d4s426	4,16	ATA CAC TGC ATC CAT ATA TAC AAG G	ACA TTG TGA AAT GAC CAC AG	HEX	49/3

Marker	Set	Primer sequence (labelled)	Primer sequence (unlabelled)	Label	Conditions
d5s432	15	GCT TAG TTA CCA CGA AGG TGA	GAC CAG ATT CCC AGT T	FAM	55/3
d5s416	11	CTG GGG CTG TTT GTC A	AGT GAA ACT CGG NCC CTA	HEX	53/2
d5s268	5	CAA TCA GGC CAT TTT TAA CTT CA	AAG GTG AGG CAA AAT GAG TGT A	FAM	53/2
d5s419	5	ATC TTT TAT TGT GGG GTG CT	TGC CCA GAC TTC TCA CCT	HEX	53/2
d5s426	5	GAT CAG GTT TCT GAA AAG TGG G	GTT CTC TTT CCA TGG TGG ATG	HEX	53/2
d5s407	5,15	GCC CCG TCT GCA ATA ACT AG	TGT TGT TCA TTG GAA GTC AAG A	FAM	58/2
d5s428	5	AAC ATC TTA GGG CAT CCT G	AAT GAT TTA AAA TAG ATT AGG AGC A	HEX	53/2
d5s409	5	GGG ATG AAG TGT GGA TAA AC	TAG GAT GGC AGT GCT CTT AG	FAM	53/2
d5s421	5	TTT ACA CCA GGT GGC CTC TC	AGA CAT GAG CCA CTG GGC	FAM	60/1.5
irf1	15	GGC CTC AAC TTC ATA ATC AA	GAG TGT ATC AGT CAG TCA GGG TGC	FAM	55/2
il9	17	GGT GGT TGA CCT CAA ATT GG	AGG CTT TCT CTA ATG CAG AG	TET	51L/2
d5s399	18	GAG TGGT ATC AGT CAG GGT GC	GGC CTC AAC TTC ATA ATC AA	HEX	53/2.5
d5s210	5	CTT TAA CAT CCT TTA ACA GC	ATG CAG AAT CTA CAA GGA CC	FAM	55/2
d5s410	5	TGC TAG TTT ATC CCA CTG TGA A	AAG GAG AAA ATC ATA TTT GGG C	FAM	53/1.5
d5s422	14	CCT TGC CTT TCT TCC TTT ATT T	GCT CTC ACT CAC TGA ACT CCA	TET	58/3
d5s400	6	ATG ATC ATG CCA CTG CAC TC	TCC TAA TTT GCT GGC TTC CCT GG	FAM	60/1.5
d5s408	5	CAA CCA AGA CAA AGG AAT CAT G	GAA CTC CTG GCC TCA AGT GA	FAM	61L/2
D6s470	15	AAG CGA TCT CAC CAT ATA CAC	ACA CTG CAA AAC GAT TAC CA	TET	55/2
d6s260	6	TTT TCA CTA TCA ATG GCA GC	TTC ATT TTC AGC AGC AAT TT	FAM	53L/2
d6s276	6	TCA ATC AAA TCA TCC CCA GAA G	GGG TGC AAC TTG TTC CTC CT	FAM	53/2
d6s273	6	GCA ACT TTT CTG TCA ATC CA	ACC AAA CTT CAA ATT TTC GG	HEX	53/2
tnfa	6	CCG GCT GTC CAG GCT TGT CC	GAT GTG GCG TCT GAG GGT TGT TTT	HEX	53/2
d6s291	8	CTC AGA GGA TGC CAT GTC TAA AAT A	GGG GAT GAC GAA TTA TTC ACT AAC T	HEX	53/2
d6s286	6	CCC AGC ATC ACC CCT AAT AC	TGC ATA TGT TGA ACC AAT CG	HEX	53/2
D6s268	15	CTA GGT GGC AGA GCA ACA T	AAA AGG AGG CA TTT TAA TCG	TET	55/3
d6s262	6	ATT CTT ACT GCT GGA AAA CCA T	GGA GCA TAG TTA CCC TTA AAA TC	HEX	53/2
d6s308	6	CCT TGG AGA GAA TTC ACG TAC A	TCT GCT GAG GGC CTA GTT TC	FAM	53/2
d6s314	5	ATG ACT TCT TTG GGT GGG C	GTG GGT AGC AAC ACT GTG GC	FAM	53/2
D6S279.3	6,19	AGC TTC AAG TGT AGG GCA AG	GAC ATT ATT GGG TCA GCT GA	TET	53/2
csr	6,19	ATT AGG CTG CAG CAA AGG AA	TAC ATT GTC GGT CTG GTC CA	FAM	58/2
d6s290	6	GTT TGC TGG ATG AGT GG	GAT TTG GTG AAT GCT CTG	HEX	53/2
d6s305	6	TGT TAT GTT TCG CCC TCC TC	GGA AGA GCA AGC AGC ACA C	FAM	53/2
d6s264	6,17	AGC TGA CTT TAT GCT GTT CCT	TTT TCC ATG CCC TTC TAT CA	FAM	51XL/2
d6s281	6	CAT CCC TGC AAT ATC AAA AA	GTT TCA GGC AAA GGC CAT AC	FAM	53/2
d7s531	7	AAA CTG TGG TCC TGG CTG	AAA CTA GAG TCC TGG CCT GA	HEX	58/2

Marker	Set	Primer sequence (labelled)	Primer sequence (unlabelled)	Label	Conditions
d7s513	7	ATG CAT TGC TAC AAC TAG CCA	GGG AGC AGG AAA GAT AGA CAG	HEX	56/1.5
d7s493	7,16	CCA GCC ATA GTT AAC TGC ACG	AGG AGA TGT ACT GTG GAA AGC A	HE	58/2
d7s629	7	AGA AGT AAA TCC TCA GTT CTC CA	CAA GGA TTT CTG TGG GAA AG	FAM	54/2.5
d7s484	7	TGC GTT TGA TCG AAG CAG A	GCT GAG CAA GGC ATT GTT T	FAM	56/1.5
d7s519	7	ACA GCC AAG CAT TTC TGC TG	ACA GAC CAG GAC TCA GCC AG	FAM	61/1.5
d7s502	7	GGA AGG TAT GTT GCG G	TAA GCC ACC AAG AAC ACC	HEX	57/2
d7s669	7	ATG CAA CCT ACC CTC AAA TG	TTT GAA TTT ACA GAA AAA ATG TGC	HEX	56/3
d7s524	7	AAG TAA TGC AAA ACA GCC TTG A	ACC CAC TGA AAA GAT TTG TGT C	FAM	56/2.5
d7s527	7,16	CTC CGC ATT GCA AAC TCA	CAT TTG AGG ATG CGC AAG	HEX	48/3
d7s486	7	AAA GGC CAA TGG TAT ATC CC	GCC CAG GTG ATT GAT AGT GC	FAM	56/1
cftr	7,16	CTG AGC GAC AGC AAA ATC AG	CTG TAG CTT TGC ATT AAA GCC A	FAM	55/3
d7s530	7	TGC ATT TTA GTG GAG CAC AG	CAG GCA TTG GGA ACT TTG	HEX	57/2
d7s684	7,16	CAG TGA GCC GAC ATT GTG	TAA TTA AGA GTA AAT GAG GCT GG	HEX	61/3
d7s483	13	CTG ACC ATN CTG GCA TAT GT	TTT TCA AAA ATT GGC AAA GA	FAM	53/3
d7s550	7,15	GCT ACG TCA ACT GAA AAG CA	ATT TGC TGC ACC AGT CAG TA	FAM	58/3
d8s504	8	GGC CAT ATG GGA ACT GTN T	AAG ACT CAG GTG GTT GAG TG	FAM	58/2
d8s503	14	TGT CTC TAA TGG TTC TTT GAC CA	TCC CTT ACA CAT CGC TCA GA	TET	58/2.5
d8s552	8	AAN GGA GAA AGG ATT GTA A	TCT CAG TGG GAA GGA AAA A	HEX	53L/2
d8s261	8	GGG AAG GTT TCA CAA AAA TTG	TTA TGG CCC AGC AAT GTG TA	FAM	53/2
d8s258	8,19	CTC CCA CGA ATC AAC TGA C	TTG ACA GGG ACC CAC G	HEX	53/3
d8s283	8	ATT CAT GTC TAG GCC ATT GC	AGA TAC AGA TGT AGA TCT CTC CG	HEX	53/2
d8s285	8	CTT ATA TGA CCT AGG CAT CAC A	CTG TTT TCC CTC CAA ACT CT	HEX	53/2
d8s260	8	AGG CTT GCC AGA TAA GGT TG	GCT GAA GGC TGT TCT ATG GA	FAM	53/2
d8s286	8	AGC TGT TTA TTT GCC CAT G	TGC TCA GTA AAT GTT TGT GAA	HEX	53/2
d8s273	8	AGT CCT TTA AAC AAC AAA ATC	AGG AAA ATG TAA ATT GCC AG	FAM	53L/2
d8s88	8	TCC AGC AGA GAA AGG GTT AT	GGC AAA GAG AAC TCA TCA GA	FAM	53L/2
d8s257	8	AAA GCC AGT GTT CTC TAA GG	CAA GCC TCT CCC TAA CAC CA	FAM	55/2
d8s556	8,19	TCT TCC ATA CAC ATG NAC G	TGC GTA GGA GTT AGA TAC ACT G	HEX	53L/3
d8s281	8	AGT ATT TTG TGC TGG GTG TGC	GGA GCG CAA TGG CAT AAT TA	FAM	53/1.5
d8s198	8	AAC CAG ATT AGG GAC AAA GA	TAG GGA CTA CAC ATG ATG GA	FAM	53/2
d8s284	8	TCT CTG TGC CTC CAT TTC CT	AGG GTT TAG GCC ACA TAG CA	HEX	53/2
d8s272	8	GCA CAC ACT CTT ACA CAC AAC G	TGG AAA ATA TGT GTT TTT GTG G	HEX	53/2
d9s144	9	AAA TAT TAT AGC AAG TTA ATT ACT GAA	GGA TAA ATA CAC TGG AAA AGA GAT	HEX	50L/2
d9s156	9	AAT CTC ACC TCC TCC ACG G	ATG GTG GTG AAT AGA GGG AAG	HEX	53/2

Marker	Set	Primer sequence (labelled)	Primer sequence (unlabelled)	Label	Conditions
IFNa	16	Unavailable	Unavailable	TET	65/3
d9s171	2	AGC TAA GTG AAC CTC ATC TCT GTC T	ACC CTA GCA CTG ATG GTA TAG TCT	TET	58/2.5
d9s147c	2	CTC CCT GCA CCC TTC CAT AA	AGT GTT CAC CCT AAT AAG CC	TET	58/2
d9s43	9,16	ATC AAG GAT ATT GTC CTG AGG A	GCT CAT TCT ATG AGG CCA GC	HEX	58/3
d9s15	9	TAA AGA TTG GGA GTC AAG TA	TTC ACT TGA TGG TGG TAA TC	HEX	53/2
d9s175	2	CCA GAG TTG AAA GAT GTT CTG A	GGC TTG TAG ACA GAG ACC CG	TET	58/2
d9s257	15	ACA GGT AAA TAC ATT CTA CCC TAC A	GTT TGA AGT GCT CTC CAG TG	FAM	55/2
d9s176	9,16	TTT CTC TTC CCC CAC ATC AC	GCT GCA TAA AAA GGG CAC AT	HEX	53/3
d9s154	15	GGG GAA AAA CAA TGG TGT TG	ATG TGA ATA TCN ACC ACG GG	TET	55/2
d9s118	9,15	TTG CTC CTC AAA GCC CGC A	GTG GCC TAG GAT TGT GGG A	HEX	58/3
d9s159	2	CTT TCT GAC GGC AGC CAG GT	AGC TGG AAT GAG TGC TGG GC	TET	58/2
d9s158	2	TCT CAA GCG ACA ACA ATC AC	GAT TTG GCT AAA ATA GGC TCA	TET	58/2
d10s189	3	CAA AAG TAA CCA TTG AGC CC	TTG ATA GAA GAA GCG ATA GAT CG	TET	53/2
d10s191	2,19	CTT TAA TTG CCC TGT CTT C	TTA ATT CGA CCA CTT CCC	TET	53/2
d10s197	3	ACC ACT GCA CTT CAG GTG AC	GTG ATA CTG TCC TCA GGT CTC C	TET	53/2
d10s220	3	TGT TGA CTG GAA GCC TTA CT	GCA AGA CTG CAT CTC AAA AT	TET	53/2.5
d10s537	14	CTA CTG TGC CTG GCT AGA AAA T	TAT GAT TAT GAG AGA GAC TTA CCT G	FAM	53/2.5
d10s201	3	AGC TCA TGG GAT GGA AGC AT	AGC TAA AAG GCT GCT GGA GA	TET	53/2.5
d10s192	3	GAA ACA AGG CTT ACC TAG ACC	TGT ATT TTA TGT GTG GCC TG	TET	53/2.5
d10s190	3,19	GTG TTT GGG TCA TGG AGA TG	AGG CAA AGC AGG AGC A	TET	61/1.5
d10s217	3,17	TTT GAA ACT ATT CAG TGC CAC C	GCT CAC TGA ATG CAG GCA G	TET	51XL/1
d10s212	10	GAA GTA AAG CAA GTT CTA TCC ACG	TCT GTG TAC GTT GAA AAT CCC	TET	53/2
d11s922	9,17	TCT TTG GCT ACA CTG GG TCC	AGC CAG ATG CTN AGA GGG T	FAM	51XL/1
TH2	16	CAG CTG CCC TAG TCA GCA C	GCT TCC GAG TGC AGG TCA CA	FAM	51/3
d11s569.2	8,17	GTG TAT TAG TCA GGG TTC	TGT CAG CCT CCA TAA TTC	TET	53/3
d11s899	9	CAG GAG GCA GAC ACA AGG AC	GAA TTG GAT TCT CTG GGG AG	FAM	53/1
d11s904	9	ATG ACA AGC AAT CCT TGA CC	CTG TGT TAT ATC CCT AAA CTG CTC A	FAM	58/2
d11s907	9	GCT TAT TGT CCA TAC CCA AA	AAA GNA CCT TAA TTT CAG GC	FAM	58/2
d11s903	1,19	AAC ACT TCG ATG TTC CTT CC	AGC TGA GAG CGC ATG TAT AA	TET	53/2
INT2	9	CTA AAG GTG CAT TTC AGT TCC C	ACA CAG CTT CCA GAG GGA GA	FAM	53/2
d11s916	1	GCT AGC CAA CGC CAT AGA TC	ATT TTG GGA CTT CTA AGC CTC C	TET	53/2
d11s901	9	ATT ACT GGC CTC TTT CTT CTG G	TGA GTG CAT TCA ACT TTG AAG G	FAM	58/2
d11s35	1	GAG ACT GGG GAC AGA AGT G	TTG TAT CGA TTA ACC AAC TTC	TET	53/2

Marker	Set	Primer sequence (labelled)	Primer sequence (unlabelled)	Label	Conditions
d11s927	1	CCC ATA ATC CCA GTT ACT CAG G	CTT GAA ACC AAA AGC CTG GA	TET	57/2
d11s925	9	AAT TCT GTC CAG ACC ACC CA	TTA GAC CAT TAT GGG GGC AA	FAM	53/2
d11s934	1	CCA GTC ACT CTG GGG TTG TA	AGG AAC CAC CGA GAA CAC AG	TET	53/2
d11s910	1	AGC TTT GCA GAC AAG GCA AG	TCC CTG CTC ATA AGT CAG CC	TET	58/2
d11s968	9	GGC TCT TGT AGT TTC TTA TCT CCT	AAG GCG GAT GCT GGA C	FAM	53/2
d12s99	10	TAG GGC AGA AGT GCC TGG	ACA TGA ACT TCT CAG AGC CAC A	HEX	58XL/3
d12s77	10	GAA GGG CAA CAA CAG TGA A	ACA TAC TTT TTT TTC TCC CC	FAM	53/2
d12s358	3	GCC TTT GGG AAA CTT TGG	GCA CAG ATG AGA TCC CGT	FAM	53/2
d12s364	10	CCA GTC CTG AAC AGG C	CAA ATC CCC TGG AAG TC	FAM	53/2
d12s62	10	ATC TTG AAG CCC TGT GCA AG	CAG AGA TTT CTT AAA TGC CT	HEX	58/3
d12s87	10	CTC CAG GCA TCA CAG GCT	AGC TCC TGG ACC TCC TCA CT	HEX	53/2
d12s368	3	GCA ACA CCT TTG TGA TGA AAA T	AGT CTG CAC AGC CTG TCC	FAM	58/1.5
d12s83	10	TTT TTG GAA GTC TAT CAA TTT GA	TAG CAG AGA AAG CCA ATT CA	HEX	53L/3
d12s43	10	AAT GTC CTT GTA CTT AGG AT	CAC TTA ATA TCT CAA TGT ATA C	FAM	53L/3
d12s92	10,19	TCC CAG TTT CCA TCA TTG	TAT AGT GTA TTG GTC AGG ATT CC	HEX	58XL/2
d12s95	10	AAG GTG CAA TGG GCT A	CAA CTG TGT GTG TTT ATA TGT G	HEX	53/3
s12s338	14	CTG TTG GCT GGA GAC ATC AG	AAA TTC AGC AGG CAA AAT GG	TET	58/3
ifng	20	TCTVTAC AAC ACA AAA TCA AA	GCC TTC CTG TAG GGT ATT AT	HEX	53/3
d12s366	10	GCT ACG ATC TTG CCA CTG	AAA ACC CAA ACA TTT CTC AG	FAM	53/2
d12s342	10	CGC TCT CAC AGT TCT GGA GG	GCC AAG GCA GAG TTT TGG AC	FAM	53/2
d12s97	10	GCT CGA ACT CCT CAG TGA AG	AGA CTC CTC ATC GTG GAA AA	HEX	53/2
d13s192	4	GGG TAA CAT AGC AAG ACC CC	GTC CGG CCT CTG TCG TGT	TET	58/1.5
d13s120	4	TGA CCT AGA AAT GAT ACT GGC A	TAA GGA GGA GGG ATT TAA ACC T	TET	58/2
d13s153	4	AGC ATT GTT TCA TGT TGG TG	CAG CAG TGA AGG TCT AAG CC	TET	58/3
d13s279	14	CGG AGG GTT CTT GTA GCA AG	GGG CCT TGT CAA CCT TCA TA	TET	58/1.5
d13s170	4	TTG CAC TGT GGA GAT AAA CAC A	TAG AAC TGG CTT TTC TTC CCC	TET	53/2
d13s122	4	TTG CAA TGA AGA ACA CAA TA	TGA ACC TAG ACT GGA ATA AAT	TET	53/3
d13s158	4	AGC TTT GAA ATG CCT AGG TA	GCT TTG ACA ATT TAG CAG CA	TET	53/2
d13s285	15	ATA TAT GCA CAT CCA TCC ATG C	AGA TAT TGA GGA AAG GCC AAA	HEX	58/3
d14s72	10	TCC TAA CAT TCT GCT ACC CA	TGT AAA GTT TTG TAC ATG GTG TAA T	TET	53/3
d14s50	10,19	AAC ACC CCT AAT TCA CCA CT	ATG ATT CCA CAA GAT GGC AG	TET	53/1
d14s80	10,20	CAT CTA CCT GCC GCA A	TAG CCA ATT TAT GGA TAC AAC TT	TET	53XL/3
d14s49	11	CAC TTT GTG CAG CTT AAA GA	CTT CAC TAG CTT TTT AAA GAC A	FAM	53/3

Marker	Set	Primer sequence (labelled)	Primer sequence (unlabelled)	Label	Conditions
d14s70	10	ATC AAT TTG CTA GTT TGG CA	AGC TAA TGA CTT AGA CAC GTT GTA G	TET	53/2
d14s75	11	TAC CCA GAT ATC GCA CAC TG	TTT TAA CAC CCA AGT CGC	FAM	53/2
d14s63	3	TGA CAA TCG GAA AGT GTA CA	CCA CAC TGT ATG CAT AGG G	FAM	53/2.5
d14s74	11	CCT GTA CCA CTA CCT GAG TTG AGT	CTT TGG CTG CCC GAA A	FAM	53/2
d14s68	10	AAG AAA TTG GAC TAA TAT TGT G	AGC TTT GGA AAT AAG ACC AG	TET	53/3
d14s51	11	GCC TGA TTT TGA GAT TCC TA	CCA CCA CCT CCT TAT ACA CA	FAM	53/2
d14s267	11	TTA ATG CCC ACT GAA TGC T	AAG GCA GCC CTG GTT T	FAM	55/2
d15s128	6	GCT GTG TGT AAG TGT GTT TTA TAT C	GCA AGC CAG TGG AGA G	TET	58/3
d15s118	7	GCT TCT AGC ACC ATG CTG G	TCC CCA ATA AAG AAG ACT GGG	TET	58/2
d15s117	7,16	AAT TCC CAG TTT GAG GAC TGA	CCC AAT GCA GCC CCT AAG	TET	49/2
d15s125	14	CTG TAG CAC CCC TGT GAG GT	TGA AGA CCG TGA TNC ACG	FAM	53/2
d15s114	6,17	CCA TGC TTG CGT GCA CTC	TGA CAG TGG ACA TTT CTC TG	TET	58/2
d15s127	6,16	CCA ACC ACA CTG GGA A	AAC AGT TGC CCA CGG T	TET	53/2
d15s207	14	ACT TCC TCA ATT CCC AAG AA	TTT TGA ACT GAG AAT GGA GC	TET	53/2
d16s423	11	TAC AAA ACA GGC TTG AAA GTC TCT	TTT GCC TGC CTA TTT GAT AAT GC	HEX	51/2.5
d16s407	5	GTG CGG CTG AAG AAT CAA TT	AAG GAA GCA TGT GGG AAA TG	HEX	53L/1.5
d16s405	12,20	CGT GGC CAG TTC TCT GCT	TGA AGG CAT TAC CCC ATT TC	FAM	53/2.5
d16s287	11	CAC CTC CAT TTT AGC ACT ATT TGG	GAG CCA AGT CCT TAT AGT GGA TAC	HEX	53/2
d16s420	12	GGT CTA AAG CAC CCA ACA TTA T	TTG GAA GAC CCA TTC TTC TG	FAM	58/1.5
d16s261	12	AAG CTT GTA TCT TTC TCA GG	ATC TAC CTT GGC TGT CAT TG	FAM	53/3
d16s411	12	GCT CTC ATC TCC AAA GGA GTT	CCT GTG TTG GTG CAT GTG TT	FAM	58/2
d16s415	11	TTA AAA AGA GAA CCA TAG GGT AA	CTG AGA AGG GAA GAT TAC TTG A	HEX	53/2
d16s320	12	AGT CTG AGA GAC ATC CAG GT	GTG ATA TCA GTC AGT CCT GTG	FAM	55/2
d16s503	11	AGT GCT CTG GAA TGA TGT GGA AG	TAA CTG GTA AAC AAG GTG AAA TTT G	HEX	53/2
d16s265	11	CCA GAC ATG GCA GTC TCT A	AGT CCT CTG TGC ACT TTG T	HEX	51/3
d16s515	12	GCA TCC GGG TCT ATA CAC AT	GTG TCT TTA ACC TGA AAC ACA CA	FAM	58/3
d16s516	12	CCT CCA GAA ACC GTG AGA TA	CTG TNT CTT TGA AGT TTT ATG ACC	FAM	55/3
d16s289	11	CAC CAC TTA TCA TTT CTT CC	AGT TGG AGG AAG AGA AGC AG	HEX	53/3
d16s422	12	CCA GTG TAA CCT GGG GGC	ACT TTC GAT TAG TTT AGC AGA ATG AG	FAM	53/2
d17s513	5	TTC ACT TGT GGG CTG CTG TC	TAA GAA AGG CTC CCA CAA GCA	TET	59/1.5
d17s786	5,20	TGA AAG TGA CAT GTT TTC CA	TTG GGC TCT TTT GTA AAG AA	TET	51XL/3
d17s799	12	ATT GCC AGC CGT CAG TT	GAC CAG CAT ATC ATT ATA GAC AAG C	HEX	53/2
inos	20	TGG TGC ATG CCT GTA GTC C	GAG GCC TCT GAG ATG TTG GTC	FAM	55XL/2

Marker	Set	Primer sequence (labelled)	Primer sequence (unlabelled)	Label	Conditions
d17s798	12	CTG CTA CCC TCC CTC AAT ACA	TAA CCA TGG GAC AGA TTC TGT T	HEX	55/1
d17s250	5	GGA AGA ATC AAA TAG ACA AT	GCT GGC CAT ATA TAT ATT TAA ACC	TET	53L/2
d17s934	12	AAG CAT GCA TAC ACA CAC AT	ACT CCT GTT TCC TAT CTG AGG	HEX	53/2.5
hox2b	5	AGT TCG GGA GTA AAA TTC TT	TGG GAG TGA TCA CTC AGT AC	TET	53/3
d17s787	5	TCC TTA GTC CCC CTT GGT G	GAT TGA TAC CTT TTT GAA GGG G	TET	53/2
d17s807	12	TCC ACC TGT AGA CCT GGT AAA	AGT GCT GCG TCT TAC AAC CT	HEX	53/2
d17s789	12	ACT CCA AAT CAA GTT TGT ACT GAG A	CTG CAT ACG AAG GGT AGG AC	HEX	58/2
d17s939	5	AGC TAT AAG TAA CCA TGT TTN TGG	ATT TGT GTT ACA GTT TAC TCG TG	TET	53/2
d17s784	5	GAG TCT CCT AAA TGC TGG GG	AGC TCC TGC ACA GTT CTT AAA TA	TET	53L/2
d18s59	12	AGC TTC TAT CCA ACA GGG GC	CTA CCA GAA TGT GAA CGA CCC	TET	58/1.5
d18s52	11	TTN CAA CAT AGG TTA TAC GCG	CCG GCC CAG TTC ATT TTC TA	TET	53/2
d18s62	12	GTG TTC TGT TAG ATT CTT TTG C	TGT GAA GGG TTG AGA ACA GA	TET	53/2
d18s53	12	AGG TCA CCT ACA ACT TTG GA	TTA CTG AAT AGC TAT CTG CAT G	TET	53/3
d18s71	12,20	ACC CGC TCA AAA GCC T	TTA ATG GAT TAT CAA GAG TGG TTC T	TET	55/1
d18s57	12	TTC AGG GTC TTT TGA AGA GG	AGA AGG CAT TAA ATT TTG CA	TET	53/2
d18s470	15	AGC TTA CCA CAA GGC ATA ACT	AGG GTA GAC TGT TAA CTG CNT TAG A	HEX	55/2
d18s64	12	AAT GAA AAG TTC TGG AAA TGG A	TTT TGA AAG CAT TTT GGA TGC	TET	53/2.5
d18s61	12,20	AAC CAA CAT AAT ATA GCA ATG G	TTC GAA CTT CGA ACC ACC C	TET	58/3
d19s221	9,16	GCA AGA CTC TGA CTC AAC AAA A	CAT AGA GAT CAA TGG CAT GAA A	TET	53/2
d19s49	9	ACT CAT GAA GGT GAC AGT TC	GTG TTG TTG ACC TAT TGC AT	TET	53/2
d19s225	9,18	ATG TAT GTT TGG TGT CCC C	GTA CAG TCG TGT GTG TGT GG	TET	53/1
d19s220	9	ATG TTC AGA AAG GCC ATG TCA TTT	TCC CTA ACG GAT ACA CAG CAA CAC	TET	53/2
d19s180	14	CTA AAT ATC CAT CAA GGA ATG	TAC TCC ATT TTC ATT CAG GT	TET	53/2
d19s210	9,17	TCA CAC TCA CTG GTC TCT CAC A	AGG CTC CGT GAG AGG GTG	TET	49/2
d20s186	13	AAC TTA GGC GTA CAG CAG CC	AAG TCA GAG ACA GGA AGC GG	FAM	57/3
d20s112	7	ATG GGT GTG CCA AAT CTC	TTC TTG TAA GTC AGA CAG CAT CA	TET	57/1.5
d20s200	7	AAC ACA GTC AAA GGC TGG	AGC TAC CTG GGC TCA AAT	TET	55/2
d20s106	13	TGA AAA CCC TCC ACC TGA AC	ACT GAG GTC ATG CAA GAG GC	FAM	58/2.5
d20s107	11	CTA CAT GAT GCC TCT TGG GA	TCA GAC AAT GGC AAA TTC CT	FAM	53/2
d20s120	13	TTT TAC TAA GGA GAC TCA ACA GGG	TTG CAC AAT GCC TGG AA	FAM	52/2.5
d20s171	7	TAT AGG TGA GGA CCC TGA GG	ACA CCA AGC CAT GTA ACC TG	TET	57/1.5
d20s93	6	TTC CTG ATC AGT CAC CAT GTA	CAG CCT GGG TGA CAG AGA A	TET	53/2

Marker	Set	Primer sequence (labelled)	Primer sequence (unlabelled)	Label	Conditions
d21s258	11	AGG CAT CTC CAA GAT TTC TAG G	CCA CGG GAC AGT GTT CAC TA	TET	55/2
d21s265	11	TTA AAG CAA TCA ATC ATG G	GGG TTC TGT GAA TAT GGG	TET	53/2
d21s219	11	CAA AAG AAA TGG CAA ACA TCT G	TGC CTC TTG ACT TTT TGG CT	TET	55/2
d21s65	3	TTA AAG CAA TCA ATC ATG G	GGG TTC TGT GAA TAT GGG	TET	53/2.5
d21s167	11,16	TTC CAT GTA CTC TGC ATT CAC A	ACA TGC CTT GCC CTG AAG	TET	53/1.5
d21s270	11	GAA ATG TTT TAA TAA ATG GTG GTT A	ACA AAG TTA TGG TCA AGG GG	TET	53/2
d21s268	13	CTA CCC GGG AGG CTG AAG	CTA CAC TGT TTT CCA AAT TGG C	TET	56/2.5
d21s1259	16	GGG ACT GTA ATA AAT ATT CTG TTG C	CAC TGG CTC TCC TGA CC	TET	58/3
d22s264	8	ATT AAC TCA TAA AGG AGC CC	CAC CCC ACC AGA GGT ATT CC	TET	53/2
d22s156	8	AGC CTG GGA GTC AGA GTG A	AGC TCC AAA TCC AAA GAC GT	TET	55/1
cyp2d	8	CCC CAC TTT TTA CTA CAC AGG C	TGA GGC AGT CAG ATG ACC TG	TET	53/2
d22s274	8	GGA GGA CTG CTT GAG TCC AG	GCT TGC TAA ATC GCT CTC TAA A	TET	53/2
dxs996	13,15	AAA TTC TTG CTT AGG CCA CTC TAG G	ACG TTG TTC TGG ATC GTA TGC TAG G	HEX	58/2
dxs999	13	GCT AAC AAC CTA GAC TTC AAC C	CAG TTT CAC AAT CTC TGC C	HEX	51/2.5
dxs451	13,15	CTT GAT CTT CTG AGG AGT GG	TTA TTC CTA GGC TTA GGA TTC	HEX	58/3
dmd	13,16	TAG CTA AAA TGT ATG AGT A	AAT AGT GTT TTC CTA AGG G	TET	49/3
dxs538	13,15	CTG ATT CAC TGT ACA ATG GT	ATG GAT AAT AAA CAG ACA GGA	TET	55/2
dxs1068	13	CCT CTA AAG CAT AGG GTC CA	CCC ATC TGA GAA CAC GCT G	TET	53/3
dxs993	13,15	AAG GAC ATT ATT TGG GTT TG	TTC ACT GGG GCC TCC TCT TC	HEX	55/2
maob	13,15	CTT CAC AGC CTC TCT CCC AG	CTT CCT ATT TCT CTC TCT GTC	TET	58/3
dxs991	11	CAC TTC AAC CAC AGA AGC CT	ATC ATT TGA GCC AAT TCT CC	TET	53/2
AR2	19	ACA GCC GAA GAA GGC CAG TTG TAT	CAG GTG CGG TGA AGT CGC TTT CCT	FAM	51/3
dxs990	13	ATA TGA CCA GTA CAA ACA TAC ACA CA	ATT CTG GAT GAT TCA CTC ATG C	TET	55/2.5
dxs1047	6	CCGGCTACAAGTGATGTCTA	CCTAGGTAACATAGTGAGACC TTG	HEX	53/2
dxs984	13	ATT TAA TGG CAG CGT ACA TGC	GGC CCC TTT ACA CCA AGC	HEX	57/2.5
dxs998	13,15	CAG CAA TTT TTC AAA GGC AA	TGT AAG AAA AAT CTT TTG TAG G	TET	55/3

APPENDIX 3 – TABLES SHOWING 1ST ROUND GENOME SCREEN RESULTS

Tables showing the results from the 1st round of the genome screen from Tamil Nadu are summarised in the tables on the following pages.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombination	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
6	Proterminus														
7	**D1S228**	32.4	114.5	49.1	65.4	0.92	42(51)	0.001	122	53.2	68.8	0.5	0	0	0
8	**D1S470**	57.2	102.7	43.6	59.1	0.93	40(36)	0	109	47.9	61.1	0.5	0	0	20.7
9	**D1S255**	66.6	88.3	37.7	50.7	0.9	38(25)	0	94.4	39.7	54.7	0.5	0	0	29.4
10	**D1S197**	78.3	109.8	45.8	64	0.96	45(37)	0	115.5	47.5	68	0.5	0	0	40
11	**D1S209**	95.9	95.3	46.2	49.1	0.58	44(30)	0	110.9	51.6	59.3	0.5	0	0	55.3
12	**D1S216**	107.2	94.2	44.7	49.4	0.66	38(30)	0	108.3	52.5	55.8	0.5	0	0.012	65.6
13	**D1S207**	117.6	120.5	64.4	56.1	0.26	51(29)	0.109	123.4	67.1	56.3	0.176	0.188	0.233	75.1
14	**D1S252**	155.1	105.5	59.5	46	0.1	53(26)	0.97	113.6	64.6	49	0.066	0.491	0.81	104.4
15	**D1S196**	186.4	86.8	41.6	45.2	0.63	34(39)	0.001	105.4	51.5	53.9	0.494	0	0.09	129.6
16	**D1S238**	206.7	122.5	51.4	71.1	0.96	58(23)	0	129	53.3	75.7	0.5	0	0.001	146.9
17	**D1S249**	225	134.2	69.9	64.4	0.36	55(44)	0.095	145.4	76.1	69.3	0.277	0.076	0.034	162.7
18	**IL10**		118	65.2	52.8	0.13	58(19)	0.402	139.4	73.9	65.5	0.306	0.056		
19	**D1S249**	241.6	94.5	55.8	38.7	**0.048**	32(44)	**1.153**	99	58	41	**0.041**	**0.653**	**0.525**	177.2
20	**D1S103**	250	105.5	57.2	48.3	0.22	47(23)	0.914	109.7	59.7	50	0.104	0.345	0.953	185
21	Proterminus	*303*													
22															
23	Proterminus														
24	**D2S281**	12.6	89.4	45.4	44.1	0.5	34(42)	0	109.6	54.8	54.8	0.5	0	0	0
25	**D2S131**	32.9	123.2	63.1	60.1	0.43	58(22)	0.018	134.8	69.3	65.5	0.355	0.03	0.042	17.3
26	**D2S165**	50.7	121	76.1	44.9	**0.002**	50(42)	**2.109**	131.3	82.1	49.2	**0.0009**	**2.103**	**1.279**	32.7
27	**D2S177**	62.8	77.2	40.6	36.6	0.37	35(14)	0.121	87	44.8	42.2	0.316	0.05	0.14	45.4
28	**D2S134**	88	92.3	44.7	47.7	0.58	40(26)	0.091	112.4	57.2	55.2	0.429	0.007	0.098	64.7
29	**D2S139**	106	136.2	74.3	61.9	0.15	63(30)	0.173	147.1	81.8	65.3	0.096	0.369	0.427	80.3
30	**IL 1α**	126.7	112.4	69.5	42.9	**0.007**	43(54)	**1.163**	125	76.2	48.8	**0.007**	**1.29**	**0.937**	98
31	**D2S160**	127.4	101.6	56	45.6	0.18	42(40)	0.387	117.7	66.5	51.2	0.1007	0.354	0.9	98.2

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombination	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
32	D2S114	147	24.3	12.4	11.9	0.5	11(6)	0.04	44.8	21.8	23	0.5	0	0.442	115.3
33	D2S142	166.3	105.3	59.2	46.1	0.12	45(43)	0.125	129.6	69.6	60	0.244	0.105	0.235	131.7
34	D2S326	184.1	122.9	66.7	56.3	0.21	58(22)	0.137	135.6	71.8	63.8	0.289	0.067	0.036	147.3
35	D2S152	194.4	112.9	51	61.9	0.83	52(30)	0.005	135.6	63.4	72.2	0.5	0	0	156.5
36	D3S11	196	127.7	57.2	70.4	0.86	64(27)	0	145.3	64.3	81	0.5	0	0	158.5
37	D2S72	202	77.5	38.2	39.3	0.5	33(32)	0	99.7	47.9	51.8	0.5	0	0	164.2
38	D2S157	212.6	106.8	47.5	59.3	0.86	54(20)	0	123	55.3	67.7	0.5	0	0	174.2
39	NRAMP	221.7	25.6	16.4	9.6	0.11	2(32)	0.413	30.2	16.4	13.8	0.315	0.05	0	182.2
40	D2S1421	225.4	53.8	20.8	33	0.94	28(11)	0	66.6	29.1	37.5	0.5	0	0	185.8
41	D2S126	228.8	86.5	39.5	47	0.74	40(27)	0	107.9	51.1	56.8	0.5	0	0	189.4
42	D2S159	236.1	149.7	66.7	83	0.89	72(33)	0	162.3	73.8	88.5	0.5	0	0	196
43	D2S206	248.3	123.1	59.1	63.9	0.61	53(37)	0.016	146.8	72.2	74.6	0.5	0	0	206.8
44	D2S125	269.5	136	76.2	59.8	**0.084**	65(31)	**1.487**	147.4	83.2	64.2	**0.028**	**0.788**	**1.217**	225.4
45	Proterminus	*284*													
46															
47	Proterminus														
48	D3S1297	2.5	129.6	67.1	62.4	0.36	57(37)	0.078	140.8	72.1	68.7	0.325	0.045	0.058	0
49	D3S1560	12.9	NO DATA												
50	D3S1263	30.4	157.5	83.3	74.2	0.26	82(18)	0.09	161.8	85.5	76.3	0.271	0.08	0.208	22.9
51	D3S1289	69	123.2	56	67.2	0.84	65(21)	0	149.3	68.1	81.2	0.5	0	0	53
52	D3S1300	79	144.2	72.1	72.1	0.5	73(22)	0	153.9	78.2	75.7	0.386	0.018	0	62.2
53	D3S1261		76.2	37.9	38.2	0.5	24(37)	0.056	82.4	42.7	39.7	0.362	0.027	0.059	78
54	D3S1271	117.7	103	55.4	47.6	0.24	46(30)	0.176	111.9	58.9	53	0.261	0.089	0.192	95.5
55	D3S1303	138.4	139.8	77.2	62.6	0.12	69(26)	0.422	151.1	81.9	69.2	0.089	0.391	0.269	113.1
56	D3S1576	156.1	102.9	48.5	54.4	0.69	42(37)	0	117.3	56.8	60.5	0.5	0	0.06	128.5
57	D3S1279	173	125.8	74.9	51	**0.019**	60(32)	**0.89**	139.1	80.5	58.6	**0.028**	**0.793**	**1.306**	143.3

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombinatio	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
58	D3S1282	186.4	97.8	51.6	46.2	0.34	39(31)	0.08	107.6	59.9	47.7	0.145	0.243	0.802	155.3
59	D3S1262	207.2	98.9	45.6	53.3	0.76	38(38)	0	109.8	51.8	58	0.5	0	0	173
60	D3S1314	218.3	90.8	45.8	45	0.5	42(13)	0	101.2	53.4	47.8	0.302	0.059	0	183.1
61	D3S1265	228	112.8	53.2	59.6	0.68	51(29)	0	124.6	59.6	65	0.5	0	0	192
62	Proterminus	*234*													
63															
64	Proterminus														
65	D4S412	3	93.8	43.6	50.2	0.73	35(32)	0	120.3	51.9	68.4	0.5	0	0	0
66	D4S403	24	5	4	1	0.19	2(1)	0.488	10.8	7.5	3.3	0.088	0.398	0	17.9
67	D4S418	43	106.2	49.1	57.1	0.75	43(36)	0	128.4	60.4	68	0.5	0	0	34.3
68	D4S405	56	121.2	59.5	61.7	0.54	47(44)	0	128.3	62	66.3	0.5	0	0.002	46
69	D4S398	71	99.3	45.8	53.5	0.76	46(29)	0.001	130.1	65.8	64.3	0.483	4E-04	0.1	59.3
70	D4S231	93	58.3	32.4	25.9	0.21	24(17)	0.186	84.4	44.1	40.3	0.39	0.017	0.058	77.9
71	D4S250	98	NO DATA												
72	D4S406	115	148.2	79.2	69	0.23	74(23)	0.074	162.3	86	76.3	0.263	0.087	0.202	96.5
73	D4S430	125	NO DATA												
74	D4S1565	149	105.3	57.7	47.6	**0.19**	45(37)	**0.409**	128.3	71.8	56.5	**0.051**	**0.581**	**0.633**	123.5
75	D4S413	157	125.5	71.3	54.2	0.076	52(41)	0.59	129.6	72.1	57.5	0.081	0.425	0.189	130.9
76	D4S415	185	137.2	57.7	79.4	0.96	64(35)	0	149.2	64.4	84.8	0.5	0	0	153.8
77	D4S408	199	NO DATA												
78	D4S426	211	61.8	24.1	37.8	0.94	21(33)	0	83.4	35.1	48.3	0.5	0	0	175.3
79	Proterminus	*225*													
80															
81	Proterminus														
82	D5S432	22	116.5	53.5	63	0.77	56(26)	0	132.7	58.1	74.6	0.5	0	0	0
83	D5S416	27.9	125.8	53.4	72.4	0.95	54(41)	0	136.2	59.4	76.8	0.5	0	0	5.6

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombinatior	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
84	D5S268	30	108.5	50.4	58.1	0.75	49(35)	0	123	56.7	66.3	0.5	0	0	7.7
85	D5S419	39.5	139.2	70.6	68.6	0.47	66(23)	0.047	142.9	72.2	70.7	0.384	0.019	0	16.4
86	D5S426	51.6	90.8	44.1	46.8	0.54	35(36)	0.001	100.1	47.8	52.3	0.5	0	0	27.3
87	D5S407	65	134.2	68.5	65.7	0.43	62(23)	0.05	147.7	73.9	73.8	0.345	0.035	0	39.3
88	D5S428	95.4	134.7	60.7	74	0.87	61(27)	0	139	63	76	0.5	0	0	63.9
89	D5S409	109.3	66.3			0.73	16(46)	0	NO DATA						85.4
90	D5S421	121.4	120	55.4	64.6	0.77	53(30)	0.001	125.6	59.9	65.7	0.5	0	0	102.5
91	IRF1		83.7	44.6	39.1	0.33	30(52)	0.035	97.3	52.5	44.8	0.208	0.144		
92	IL9														
93	D5S399		123.4	59.9	63.5	0.61	47(37)	0	126.6	62.8	63.8	0.5	0		
94	D5S210	143	102.2	43.5	58.7	0.92	37(47)	0	111	47.7	63.3	0.5	0	0	104.1
95	D5S410	156	95.7	48	47.7	0.5	38(39)	0.035	104	53	51	0.359	0.028	0.121	115.8
96	D5S422	163.9	155.2	89.1	66.1	**0.038**	75(24)	**1.24**	163.2	93.3	69.9	**0.024**	**0.845**	**0.787**	123.2
97	D5S400	174.3	81.8	43.7	38.1	0.33	39(21)	0.114	103.7	54.7	49	0.251	0.098	0.059	*136.5*
98	D5S408	195.8	102.5	59.3	43.2	**0.069**	47(38)	**0.744**	122.8	71.5	51.3	**0.03**	**0.766**	**0.864**	151.2
99	Proterminus	*201*													
100															
101	Proterminus														
102	D6S470	17.7	131	71.5	59.5	0.17	60(31)	0.51	143.3	77.4	65.9	0.153	0.227	0.299	0
103	D6S260	29.6	115.8	59.2	56.7	0.43	60(14)	0.018	131.1	66	65.1	0.378	0.021	0.025	10.8
104	D6S276	44.9	122	58	64	0.67	63(22)	0	140.2	70.5	69.7	0.465	0	0.025	24.3
105	D6S273		92.7	54.2	38.4	**0.059**	35(34)	**0.706**	100	57.7	42.3	**0.046**	**0.618**	**0.075**	24.9
106	TNFa	46	122.7	66	56.7	0.21	52(34)	0.141	129	67.9	61.1	0.232	0.117	0.074	25.4
107	D6S291	49.8	113.6	55.2	58.4	0.57	47(39)	0.005	136.9	64.9	72	0.5	0	0.046	29.1
108	D6S286	90	108.3	48.7	59.7	0.83	42(40)	0	115	51	64	0.5	0	0	60.2
109	D6S268	114	5	1	4	0.81	3(1)	0	7.6	1.6	6	0.5	0	0	80.3

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombination	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
110	D6S262	129.8	118	55.6	62.4	0.71	55(30)	0	130	60.7	69.3	0.5	0	0	94.2
111	D6S308	145.5	94.3	44.1	50.2	0.7	37(37)	0	106.4	51.7	54.7	0.5	0	0	107.1
112	D6S314	144.4	118.5	59.8	58.7	0.5	56(32)	0.006	131.5	66.5	65	0.439	0.005	0.017	108.2
113	D6S279	145.5	17.8	7.8	10	0.6	11(1)	0	17	7.5	9.5	0.5	0	0.017	108.2
114	D6S311	148.2	NO DATA												
115	ESR		71.2	44.9	26.2	**0.021**	30(19)	**1.165**	88.2	53.9	34.3	**0.019**	**0.939**	**0.698**	113.5
116	D6S290	155.3	92.8	43.6	49.2	0.7	42(21)	0	109.4	53.2	56.2	0.5	0	0.249	117.5
117	D6S305	166.6	86.7	37.9	48.8	0.86	37(28)	0	100.1	44.1	56	0.5	0	0.122	127.8
118	D6S264	179.1	103.4	51.7	51.7	0.5	39(47)	0.031	111	58.5	52.5	0.287	0.069	0.407	139
119	D6S281	201.1	100	57.9	42.1	0.08	47(22)	0.579	107.4	60.1	47.3	0.086	0.405	0.824	157.6
120	Proterminus	*207*													
121															
122	Proterminus														
123	D7S531	4.8	92.7	45.6	47.1	0.54	33(19)	0	99.4	49	50.4	0.5	0	0	0
124	D7S513	20	98.5	47.9	50.6	0.58	46(18)	0.021	106.8	53.3	53.5	0.5	0	0.197	13.4
125	D7S493	35	128.9	60	68.9	0.76	56(34)	0.04	136.5	66.3	70.2	0.5	0	0.036	26.7
126	D7S629	36.7	109.3	52.2	57.1	0.65	47(29)	0	119.9	57.6	62.3	0.5	0	0.217	28.4
127	D7S484	55.6	81	41.7	39.3	0.46	33(26)	0.195	86.9	46.2	40.7	0.216	0.134	0	44.7
128	D7S519	70.5	68.3	31.4	36.9	0.69	32(10)	0	85	40	45	0.5	0	0.000	57.9
129	D7S502	79.6	119.2	58.3	60.9	0.54	60(16)	0	126.4	63.7	62.7	0.5	0	0.028	66.3
130	D7S669	90.9	55.8	25.6	30.2	0.7	20(23)	0	60.5	27.8	32.7	0.5	0	0.011	76.6
131	D7S524	98	108.7	51.7	57	0.68	52(28)	0.005	124.7	60	64.7	0.5	0	0.223	83.3
132	D7S527	109	25.3	12.3	13	**0.5**	10(9)	**0.008**	35.1	17.1	18	**0.435**	**0.006**	**1.233**	93.3
133	D7S692	XXX	67.2	41	25.7	**0.032**	27(39)	**0.95**	100.1	59	40.9	**0.025**	**0.837**	**0.065**	XXX
134	D7S486	125.3	86.2	38.9	47.2	0.81	34(29)	0	101	46.2	54.8	0.5	0	0.062	107.4
135	CFTR	125.5	73	45.1	27.9	**0.022**	19(60)	**3.149**	79.4	49.7	29.7	**0.001**	**1.937**	**0.059**	107.6

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombination	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
136	D7S550	136.4	117.7	60.4	57.3	0.43	42(47)	0.233	131.2	66.9	64.3	0.249	0.1	0.072	117.7
137	D7S684	149.6	72	36.2	35.8	0.5	30(24)	0.081	83.3	45	38.3	0.218	0.132	0.585	129.4
138	D7S483	167.6	119.8	65.7	54.1	0.18	59(21)	0.214	127.3	69.3	58	0.15	0.234	0.819	145.2
139	D7S550	180.8	113.3	68.1	45.2	**0.019**	49(37)	**1.413**	128.1	76.2	51.9	**0.0095**	**1.197**	**0.757**	157
140	Proterminus	*206*													
141															
142	Proterminus														
143	D8S504	0	102.2	54	48.3	0.35	47(25)	0.357	113.9	59.8	54.1	0.361	0.027	0.313	0
144	D8S503	15.5	107.7	53.5	54.2	0.5	55(27)	0	136	62.7	73.3	0.5	0	0.002	13.7
145	D8S552	25.8	63	33.2	29.8	0.35	29(19)	0.012	85.9	43.2	42.7	0.462	0.002	0.019	23.1
146	D8S261	35	110	55.3	54.7	0.5	41(50)	0	118.1	59.4	58.7	0.5	0	0.006	31.6
147	D8S258	40.3	119.6	58.2	61.4	0.57	45(44)	0	125.3	62	63.3	0.5	0	0	36.6
148	D8S283	60	83.6	45.9	37.7	0.22	37(25)	0.298	93.8	53.5	40.3	0.081	0.423	0.003	53.5
149	D8S285	70.6	104.7	46.8	57.9	0.84	51(21)	0	126.7	55	71.7	0.5	0	0	63.2
150	D8S260	78.8	133.2	66.4	66.8	0.5	66(26)	0.018	146.2	75	71.2	0.315	0.05	0	70.9
151	D8S286	93.5	106.3	62.6	43.8	**0.039**	47(31)	**1.148**	118.4	70.2	48.2	**0.01**	**1.161**	**0.654**	83.9
152	D8S273	101	120.3	65.7	54.6	0.18	55(29)	0.273	134.9	71.4	63.5	0.202	0.151	0.675	90.9
153	D8S88	102	85.7	46.4	39.3	0.26	40(15)	0.257	85.8	47.1	38.7	0.14	0.254	0.726	91.9
154	D8S257	110.3	122.5	69.3	53.2	**0.087**	52(36)	**0.796**	138.2	78.2	60	**0.046**	**0.619**	**0.787**	99.6
155	D8S556	116	72.7	44.8	27.9	**0.028**	21(49)	**1.678**	82.6	50.1	32.5	**0.014**	**1.041**	**0.535**	105
156	D8S281	122.6	73.4	42.2	31.2	0.12	13(61)	0.337	84.8	47.1	37.7	0.126	0.287	0.231	111.2
157	D8S198	130	96.5	47.8	48.7	0.5	47(32)	0.161	111.7	58.4	53.3	0.361	0.028	0.265	118.1
158	D8S284	142.7	115.5	56.3	59.2	0.57	56(33)	0	129.1	64.3	64.8	0.5	0	0	129.5
159	D8S272	152.5	64.1	32.8	31.3	0.5	20(37)	0.058	86.2	45.1	41.1	0.239	0.109	0	138.5
160	Proterminus	*154*													
161															

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombinatio	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
162	Proterminus														
163	**D9S144**	15	102.8	49.2	53.7	0.62	32(31)	0.001	114.5	56.3	58.2	0.5	0	0.02	0
164	**D9S156**	29.5	113.6	53.4	60.2	0.71	52(33)	0	130.8	62	68.8	0.5	0	0	12.9
165	**IFNa**		58.8	27.8	31	0.6	25(33)	0	78.4	40.9	37.5	0.385	0.018		
166	**D9S171**	42	85	38.2	46.8	0.78	21(57)	0	92.4	41.1	51.3	0.5	0	0	24.1
167	**D9S147E**	52	112.3	59.8	52.5	0.28	45(36)	0.103	125.9	66	59.9	0.375	0.02	0.076	33.3
168	**D9S43**	53	82.5	43.2	39.3	0.37	37(22)	0.086	98.5	51.2	47.3	0.38	0.02	0.108	34.3
169	**D9S15**	62	112	57	55	0.46	51(32)	0.398	130.7	64.4	66.3	0.5	0	0.524	42.6
170	**D9S175**	68.8	131.3	64.4	66.9	0.53	57(33)	0.355	146.4	75.4	71	0.363	0.027	0.523	49
171	**D9S257**	90	79.8	49.9	29.9	**0.015**	31(24)	**1.245**	109.5	66.8	42.7	**0.0069**	**1.319**	**1.124**	67
172	**D9S176**	104.9	106	63.2	42.8	**0.025**	36(46)	**0.886**	118	67.5	50.5	**0.053**	**0.562**	**1.094**	80.2
173	**D9S154**	125	88.3	40.8	47.5	0.74	36(29)	0	107.6	51.2	56.4	0.5	0	0.022	97.4
174	**D9S118**		151	80	71	0.23	75(24)	0.198	156.7	81.9	74.8	0.239	0.11		
175	**D9S159**	142.7	105.5	51.8	53.7	0.54	40(40)	0.003	125.9	64.6	61.3	0.363	0.026	0	112.8
176	**D9S158**	163	20.8	10.4	10.4	0.5	9(10)	0.132	34.9	16.9	18	0.5	0	0.032	130.1
177	Proterminus	*175*													
178															
179	Proterminus														
180	**D10S189**	17.3	108.9	53.7	55.3	0.54	51(46)	0	133.8	65	68.8	0.5	0	0	0
181	**D10S191**	36.3	157.8	70.5	87.3	0.9	79(21)	0	165.3	75.6	89.7	0.5	0	0	16.4
182	**D10S197**	50.5	126.5	71.2	55.3	0.091	50(47)	0.395	134	74	60	0.127	0.283	0.073	29
183	**D10S220**	72.5	128.7	59.2	69.4	0.79	61(29)	0	145	63.8	81.2	0.5	0	0	47.6
184	**D10S537**	93.8	134.7	67.3	67.4	0.5	61(33)	0.013	147.7	75.4	72.3	0.315	0.05	0.001	65.7
185	**D10S201**	105.9	102.5	55.8	46.7	0.21	54(15)	0.226	113.4	59.7	53.7	0.202	0.151	0.011	71.4
186	**D10S192**	131.2	96.4	52.8	43.6	0.21	41(32)	0.458	118.6	62.3	56.3	0.254	0.096	0.442	96.7
187	**D10S190**	147.2	140.8	75	65.8	0.22	63(35)	0.371	143.4	78.7	64.7	0.096	0.371	0.413	110.8

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombinatio	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
188	D10S217	167.2	113.2	60.9	52.4	0.25	47(34)	0.297	123.8	66.1	57.7	0.17	0.197	0.019	127.9
189	D10S212		89.2	43.9	45.2	0.54	32(45)	0.002	105.8	53.5	52.3	0.468	0.001	0.071	140
190	Proterminus	*178*													
191															
192	Proterminus														
193	D11S922	3.2	111	51.8	59.2	0.75	52(14)	0	141.2	67.9	73.3	0.5	0	0	0
194	TH.2	8	120.8	55.2	65.6	0.79	50(50)	0	138.9	62.2	76.7	0.5	0	0	4.6
195	D11S569	22	58.7	30.8	27.9	0.4	35(21)	0.174	81.6	42.8	38.8	0.27	0.082	0.066	17.1
196	D11S899	26.5	NO DATA												
197	D11S904	37	139.1	70.3	68.8	0.47	66(38)	0.092	159.6	75.1	84.5	0.5	0	0.101	30.4
198	D11S907	46.9	95.8	52.6	43.2	0.21	42(32)	0.263	110.8	58.7	52.1	0.299	0.06	0.078	39.5
199	D11S903	59.5	80.2	38.8	41.5	0.59	24(44)	0.008	88.9	43.1	45.8	0.5	0	0	50.8
200	INT2	74	99	53.4	45.6	0.24	48(24)	0.203	113.4	62.7	50.7	0.117	0.308	0.012	63.7
201	D11S916	80.1	121	63.2	57.8	0.32	59(18)	0.063	131.2	69	62.2	0.26	0.09	0.019	69.5
202	D11S901	89.8	125.7	71.8	53.9	0.063	47(45)	0.663	137.3	76.3	61	0.084	0.412	0.174	78.4
203	D11S35	97	99.3	55.6	43.8	0.13	34(41)	0.458	106.5	58.9	47.6	0.103	0.348	0.569	85.1
204	D11S927	108.7	81.3	47.4	33.9	0.073	40(17)	0.365	117.3	67	50.3	0.054	0.564	0.917	95.7
205	D11S1356	118.6	NO DATA												
206	D11S925	123.5	88.4	42.8	45.7	0.58	39(26)	0.285	98.6	47.6	51	0.5	0	0.357	108.8
207	D11S934	131.7	111.3	59.7	51.7	0.25	45(30)	0.14	110	59	51	0.225	0.124	0.362	116.4
208	D11S910	145.6	105.3	50.8	54.6	0.62	46(29)	0	125.7	61.5	64.2	0.5	0	0.025	128.8
209	D11S968	152.8	127.1	61.8	65.3	0.61	53(41)	0	133.6	65.3	68.3	0.5	0	0.001	135.5
210	Proterminus	*160*													
211															
212	Proterminus														
213	D12S99	13.9	140.6	69	71.6	0.57	67(30)	0	152.1	74.6	77.5	0.5	0	0	0

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombination	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
214	D12S77	21.8	145.1	78.4	66.7	0.18	61(37)	0.211	153.5	84	69.5	0.151	0.231	0.15	7.4
215	D12S358	27.2	103	53.1	49.9	0.38	57(32)	0	135.8	63.1	72.7	0.5	0	0	12.5
216	D12S364	31.7	152.7	86.8	65.9	0.052	78(17)	0.483	163.2	91.7	71.5	0.075	0.448	0.039	16.8
217	D12S62		137.5	74.1	63.4	0.2	65(31)	0.124	156.9	83.2	73.7	0.286	0.069		
218	D12S87	53.3	105.1	49.4	55.7	0.69	42(51)	0	129.2	64.4	64.8	0.5	0	0	35.1
219	D12S368	67.3	102.3	49.4	53	0.58	50(29)	0	134.7	69.8	64.9	0.377	0.02	0.054	47.6
220	D12S83	76.5	127	69.3	57.7	0.16	55(33)	0.196	140	75	65	0.247	0.102	0.185	56.1
221	D12S43	80	123.8	70.5	53.3	**0.074**	59(26)	**0.663**	144.9	85	59.9	**0.016**	**0.992**	**1.215**	59.5
222	D12S92	84.2	*41.7*	22.8	18.9	**0.32**	20(13)	**0.167**	59.7	31.5	28.2	**0.294**	**0.064**	**1.094**	63.5
223	D12S95	97.7	82.8	48.8	34	**0.06**	39(14)	**0.565**	99.3	58.8	40.5	**0.037**	**0.689**	**1.468**	75.6
224	D12S338	113.3	147.7	78.6	69.1	0.25	68(34)	0.151	155.2	83.4	71.8	0.171	0.196	0.244	89.3
225	IFN GAMMA		0	0	0	0	0	0	9.4	8.4	1	0.003	1.59		
226	D12S366	133.8	127.1	74.3	52.8	**0.03**	51(44)	**1.146**	139.9	78.2	61.7	**0.037**	**0.692**	**0.461**	106.8
227	D12S342	145.7	119.2	67.6	51.7	0.084	50(41)	0.522	130.5	72.5	58	0.056	0.549	0.452	*117.6*
228	D12S97	160.9	89.6	48.9	40.7	0.23	31(52)	0.283	112.3	59.9	52.4	0.206	0.146	0.315	131
229	Proterminus	*179*													
230															
231	Proterminus														
232	D13S192	18	151.8	72.3	79.6	0.69	78(15)	0	166.5	81.5	85	0.5	0	0	0
233	D13S120	21	126	65.2	60.8	0.36	54(37)	0.002	134.6	68.3	66.3	0.5	0	0.024	2.9
234	D13S153	52	145	84.4	60.6	**0.027**	67(24)	**1.152**	156.2	90.5	65.7	**0.021**	**0.897**	**1.447**	27.9
235	D13S279	65	120.1	66.7	53.4	0.14	51(36)	0.131	133.8	72.4	61.4	0.183	0.178	0.76	39.6
236	D13S170	77	135.3	71.1	64.2	0.3	70(29)	0.103	153.6	81.6	72	0.252	0.097	0.202	50.4
237	D13S122	88	113.7	52.4	61.4	0.77	47(37)	0	125.7	61	64.7	0.5	0	0.156	60.4
238	D13S158	99	126.8	66.2	60.7	0.33	54(44)	0.024	137	71.7	65.3	0.285	0.07	0.485	70.4
239	D13S285	127	123.3	66.4	57	0.21	58(27)	0.118	139.8	73.9	65.9	0.29	0.066	0.439	93.3

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombination	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
240	Proterminus	*130*													
241															
242	Proterminus														
243	**D14S72**	2.1	127.1	68.3	58.8	0.21	56(34)	0.727	157.1	80.8	76.3	0.376	0.021	0.076	0
244	**D14S50**	8	108.7	52.3	56.3	0.61	47(28)	0	127.3	61.3	66	0.5	0	0	5.6
245	**D14S80**	20.6	132.6	67.5	65.1	0.47	51(44)	0.176	140	69.2	70.8	0.5	0	0	13
246	**D14S49**	30	156.4	70.1	86.3	0.89	66(43)	0	159.8	72.8	87	0.5	0	0	25.5
247	**D14S70**	32.9	84.8	38.1	46.8	0.78	33(42)	0	123.5	54.9	68.6	0.5	0	0	28.3
248	**D14S75**	36.3	141.5	67.1	74.4	0.69	63(32)	0	148.2	69.9	78.3	0.5	0	0	31.6
249	**D14S63**	59	69.8	34.9	34.9	0.5	25(28)	0	91.7	43	48.7	0.5	0	0	50.7
250	**D14S74**	76.4	125.3	66.9	58.4	0.26	57(27)	0.209	136	73.7	62.3	0.162	0.211	0.052	65.8
251	**D14S68**	86.3	137.6	70.4	67.2	0.43	68(21)	0.195	164.1	78.5	85.6	0.5	0	0	74.9
252	**D14S51**	109.6	141	81	60	**0.038**	73(29)	**0.431**	154.2	85.4	68.8	**0.091**	**0.388**	**0.048**	94.5
253	**D14S267**	112.6	122.8	54.3	68.6	0.88	57(22)	0	138.8	60.3	78.5	0.5	0	0.015	97.4
254	Proterminus	*134*													
255															
256	Proterminus														
257	**D15S128**	6.1	126.2	60.9	65.2	0.64	57(18)	0.087	130.1	64.4	65.7	0.5	0	0.09	0
258	**D15S118**	32.2	82.2	40.5	41.7	0.5	39(25)	0.05	96.6	48.3	48.3	0.5	0	0.035	21.6
259	**D15S117**	50.8	137.7	79.1	58.5	**0.044**	62(32)	**0.435**	145.5	83.3	62.2	**0.048**	**0.601**	**0.49**	37.7
260	**D15S125**	63.8	129.1	65.3	63.8	0.46	56(38)	0	145.3	73.1	72.2	0.451	0.003	0.052	49.4
261	**D15S114**	72.3	79.8	43.5	36.3	0.25	28(41)	0.407	92.6	50.3	42.3	0.177	0.187	0.04	57.3
262	**D15S127**	84.8	99.7	48.6	51.1	0.58	43(27)	0	120	59.7	60.3	0.39	0.017	0	68.5
263	**D15S207**	100.8	103.4	48.2	55.2	0.72	45(39)	0	139	66.8	72.2	0.5	0	0	82.6
264	Proterminus	*112*													
265															

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombinatio	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
266	Proterminus														
267	D16S423	8.4	87.8	41.2	46.6	0.67	44(20)	0.051	87	39.8	47.2	0.5	0	0.158	0
268	D16S407	16.7	82	38.3	43.7	0.67	36(15)	0	100.6	48.1	52.5	0.5	0	0.089	7.7
269	D16S405	27	76.8	36.9	39.9	0.59	32(27)	0.015	110.4	58.1	52.3	0.249	0.01	0.079	17.1
270	D16S287	32	126.8	64.8	62	0.46	55(40)	0.003	136.9	71.4	65.5	0.336	0.039	0.129	21.9
271	D16S420	43.2	41.3	22.9	18.4	0.32	14(20)	0.275	69.9	38	31.9	0.211	0.14	0.195	32.1
272	D16S261	53	80.8	41.8	38.9	0.41	24(53)	0.038	92.1	46.9	45.2	0.381	0.02	0.216	41.1
273	D16S411	57.8	112.7	58.2	54.5	0.39	47(40)	0.275	130.7	69.7	61	0.148	0.238	0.123	45.7
274	D16S415	65.6	115.3	54.8	60.5	0.68	47(47)	0	130.7	64.5	66.2	0.5	0	0.037	53
275	D16S320	74	139.5	70.8	68.7	0.47	64(19)	0.015	162.4	83.8	78.6	0.326	0.044	0.133	60.8
276	D16S503	81.8	126.7	65	61.7	0.43	60(35)	0.17	148.4	77.2	71.2	0.267	0.084	0.024	68.1
277	D16S265	90.2	155.3	77.3	78	0.5	76(24)	0.001	154	76.5	77.5	0.472	0.001	0.373	75.9
278	D16S515	90.3	126.3	70.1	56.3	**0.12**	50(49)	**0.498**	138.7	78.5	60.2	**0.05**	**0.576**	**0.414**	76
279	D16S516	98.3	82.3	53.7	28.6	**0.004**	36(36)	**3.047**	99.8	61.5	38.3	**0.007**	**1.312**	**1.98**	83.4
280	D16S289	102	136.1	77.3	58.8	**0.061**	56(42)	**1.63**	142.9	84.1	58.8	**0.016**	**0.992**	**2.34**	87
281	D16S422	109.1	137.8	84.5	53.4	**0.005**	67(27)	**1.918**	155.4	92.9	62.5	**0.0039**	**1.541**	**1.357**	93.7
282	Proterminus	*134*													
283															
284	Proterminus														
285	D17S513	11	78.8	38.6	40.2	0.55	20(49)	0	87.3	43.6	43.7	0.5	0	0	0
286	D17S786	18.1	103.1	51.3	51.8	0.5	51(26)	0	126.5	65.6	60.9	0.414	0.01	0.017	6.7
287	D17S799	32.8	140.8	68.9	72	0.57	63(33)	0	147.2	71.5	75.7	0.5	0	0	19.7
288	iNOS(sibmap)		20.2	12.7	7.4	0.18	2(23)	0.261	23.9	15.2	8.7	0.081	0.425		
289	D17S798	53.9	92.8	36.6	56.2	0.98	37(33)	0	109.3	44.6	64.7	0.5	0	0	*37.6*
290	D17S250	60	127.7	59.6	68.1	0.76	55(31)	0	130	61.7	68.3	0.5	0	0	43.4
291	D17S934	63.7	125	56	69	0.84	56(33)	0	136.5	62.5	74	0.5	0	0	47

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombination	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
292	**HOX2B**	67	90.5	52.7	37.8	**0.069**	26(51)	**1.171**	93.3	56	37.3	**0.0167**	**0.985**	**0**	50.2
293	**D17S787**	75.7	107.7	55.6	52.1	0.42	46(33)	0.007	117	58.7	58.3	0.465	0.002	0	58.3
294	**D17S807**	87.6	145.8	68	77.9	0.77	70(25)	0	157.1	73.1	84	0.5	0	0	69.1
295	**D17S789**	90.8	127.1	58.8	68.3	0.79	52(47)	0.009	144.1	68.7	75.4	0.5	0	0	72.2
296	**D17S939**	107	90.3	43.9	46.4	0.58	39(14)	0	93.2	46.9	46.3	0.453	0.003	0	86.4
297	Proterminus	*133*													
298															
299	Proterminus														
300	**D18S59**	0.1	127.6	68.2	59.4	0.24	52(36)	0.14	145.3	78.8	66.5	0.169	0.199	0.66	0
301	**D18S52**	8.3	56.3	29.2	27.2	0.45	23(22)	0.07	61.4	32.7	28.7	0.248	0.101	0.165	7.6
302	**D18S62**	17.7	90.7	43.9	46.8	0.58	40(32)	0	120.9	61.4	59.5	0.5	0	0.022	16.3
303	**D18S53**	40.4	94.8	42.1	52.6	0.82	53(29)	0	118.1	57.1	61	0.5	0	0.175	35.4
304	**D18S71**	42.8	NO DATA												
305	**D18S57**	63.2	115.7	56.7	59	0.57	51(34)	0.193	129.1	67.4	61.7	0.45	0.003	0.537	54.6
306	**D18S470**	71.3	124.8	67.9	56.9	0.18	67(12)	0.717	140.2	78.9	61.3	0.054	0.559	0.743	62.1
307	**D18S64**	83	79.9	39.8	40.2	0.5	33(28)	0.134	113	58.7	54.3	0.344	0.035	0.056	72.7
308	**D18S61**	102.8	119	56.8	62.2	0.68	59(33)	0	136.2	64.7	71.5	0.5	0	0.041	89.7
309	Proterminus	*128*													
310															
311	Proterminus														
312	**D19S221**	35.5	96.2	47	49.2	0.54	40(24)	0.026	102.8	47	55.8	0.5	0	0.006	0
313	**D19S49**	49	81.7	39.1	42.6	0.59	37(31)	0	107.9	49.5	58.4	0.5	0	0	12.1
314	**D19S225**	55.9	95.8	52.1	43.8	0.21	41(33)	0.416	126.6	69.8	56.8	0.149	0.236	0.058	18.6
315	**D19S220**	61.4	91.3	41.8	49.6	0.77	46(16)	0	123	58.7	64.3	0.5	0	0	23.8
316	**D19S180**	91	108.7	52.3	56.5	0.61	44(40)	0	127	60.4	66.6	0.5	0	0	47.8
317	**D19S210**	104.9	126.5	62.9	63.6	0.5	51(44)	0.006	138.5	67.6	70.9	0.5	0	0	60.2

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombinatio	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
318	Proterminus	*110*													
319															
320	Proterminus														
321	**D20S186**	33.2	65	28	37	0.84	28(13)	0	72.9	30.9	42	0.5	0	0	0
322	**D20S112**	39.3	109.5	56.3	53.2	0.42	54(16)	0.093	118.7	60.7	58	0.318	0.049	0	5.8
323	**D20S200**	49.7	98.5	45.6	52.9	0.73	41(23)	0	114.4	52.3	62.1	0.5	0	0.014	15.3
324	**D20S106**	50.2	65	32	33	0.5	26(24)	0.012	84.6	44.6	40	0.306	0.056	0.037	15.8
325	**D20S107**	54.9	126	67.9	58.1	0.24	63(24)	0.246	134.1	74.4	59.7	0.109	0.329	0.17	20.3
326	**D20S120**	82.1	97	45	52	0.73	39(23)	0	101	46	55	0.5	0	0	42.7
327	**D20S171**	94.4	98.3	57.1	41.2	0.065	47(21)	0.45	111	62.5	48.5	0.074	0.454	0.173	53.8
328	**D20S93**	98	142.2	57.8	84.3	0.99	66(24)	0	x	x	x	x	x		
329	Proterminus	*130*													
330															
331	Proterminus														
332	**D21S258**	8	136.9	70	66.9	0.43	60(39)	0.076	148.7	71.6	77.1	0.5	0	0	0
333	**D21S265**	24.1	142.7	69.6	73.1	0.6	71(31)	0	158	76	82	0.5	0	0	14.1
334	**D21S219**	34	90.3	43.8	46.5	0.58	31(52)	0	108.1	53.1	55	0.5	0	0	23.2
335	**D21S65**	34	79.3	34.4	44.9	0.85	37(16)	0	91	41.9	49.1	0.5	0	0	23.4
336	**D21S167**	41.3	112.7	57.8	54.9	0.42	53(30)	0	132.2	64	68.2	0.5	0	0	30.1
337	**D21S270**	41.3	129.7	65.4	64.2	0.5	51(49)	0.006	137	68	69	0.5	0	0	30.3
338	**D21S268**	44.3	99	46	53	0.73	42(15)	0	104.6	49.6	55	0.5	0	0	33
339	**D21S1259**	57.7	54.4	26.7	27.7	0.5	22(36)	0.035	82.3	45.4	36.9	0.163	0.21	0.033	45
340	**D21S1260**		NO DATA												
341	Proterminus	*66*													
342															
343	Proterminus														

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1			FIRST SCREEN												
2			GAS						SIBPAIR (Global Binning)					MAPMAKER SIBS	Cumulative
3	LOCUS	Cumulative	IBD				MLS		IBD				MLS	MLS	Recombination
4		Recombinatio	no.alleles	1	0	p value	no.alleles	score	no.alleles	1	0	p value	score	score	Fraction
5		Fraction					(ambiguous	score							from mapsibs
344	D22S264	3	114.7	56.8	57.9	0.5	43(49)	0	123.3	62.1	61.2	0.5	0	0	0
345	D22S156	16	NO DATA												
346	CYP2D	33	94.3	46.9	47.4	0.5	33(50)	0.012	101.9	53.2	48.7	0.294	0.06	0.111	24.3
347	D22S274	45.5	105.5	51.8	53.7	0.54	52(25)	0.028	132.7	67.8	64.9	0.365	0.026	0.091	35.5
348	Proterminus	66													
349															
350	Proterminus														
351	DXS996	11	37.2	19.7	17.4	0.43	x	x	37	19.7	17.3	0.369	0.024	0.01	0
352	DXS999	29	50.7	25.3	25.3	0.5	x	x	52.8	26.8	26	0.462	0.002	0.143	15.6
353	DXS451	39.5	24.3	12.9	11.4	**0.5**	x	x	31.5	20.2	11.3	**0.05**	**0.586**	**0.171**	25.2
354	DMD		14.3	7.4	6.9	0.5	x	x	15	7.3	7.7	0.5	0		
355	DXS1068	56.2	58	30.2	27.8	0.4	x	x	57	29.7	27.3	0.356	0.03	0	39.8
356	DXS1058	56.4	NO DATA												
357	DXS538	59	43.5	22.2	21.3	0.5	x	x	49.6	22.6	27	0.5	0	0	42.5
358	DXS993	62	27.2	12.7	14.4	0.58	x	x	37.5	17.8	19.7	0.5	0	0	45.4
359	MAOB	75	71	31.8	39.2	0.8	x	x	72.7	34	38.7	0.5	0	0	57.1
360	DXS991	92	70.3	31.7	38.7	0.76	x	x	77.5	35	42.5	0.5	0	0	71.9
361	AR.2		NO DATA												
362	DXS990	110	66.8	32.1	34.8	0.55	x	x	66	31.7	34.3	0.5	0	0.164	87.5
363	DXS1047	160	67.2	33.9	33.2	0.5	x	x	67	33.3	33.7	0.5	0	0	124.7
364	DXS984	171	52	29.2	22.8	0.2	x	x	51	28.7	22.3	0.179	0.184	0	134.7
365	DXS998	195	63.3	34.2	29.1	0.31	x	x	65.1	34.8	30.3	0.348	0.033	0	154.8
366	Proterminus														